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Conservation of Signaling Pathways of Xenobiotic-Sensing Orphan Nuclear Receptors, Chicken Xenobiotic Receptor, Constitutive Androstane Receptor, and Pregnane X Receptor, from Birds to Humans

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Chicken xenobiotic receptor, pregnane X receptor, and constitutive androstane receptor are orphan nuclear receptors that have recently been discovered to regulate drug- and steroid-mediated induction of hepatic cytochromes P450 (CYP). This induction is part of an adaptive response involving numerous genes to exposure to drugs and chemicals and has major clinical and toxicological implications. Here we report experiments in the chicken hepatoma cell line LMH that suggest evolutionary conservation of the signaling pathways triggered by pregnane X receptor, constitutive androstane receptor, and chicken xenobiotic receptor. Thus, the phenobarbital-inducible enhancer units of the mouse Cyp2b10, rat CYP2B2, and human CYP2B6 genes were activated in reporter gene assays by the same compounds that activate the chicken CYP2H1 phenobarbital-inducible enhancer units. Chicken xenobiotic receptor, pregnane X receptor, and constitutive androstane receptor all bound to the CYP2H1 phenobarbital-inducible enhancer units in gel-shift experiments. In CV-1 cell transactivation assays, mammalian pregnane X receptors activate the chicken phenobarbital-inducible enhancer units to the same extent as does chicken xenobiotic receptor, each receptor maintaining its species-specific ligand spectrum. To assess the reported role of protein phosphorylation in drug-mediated induction, we treated LMH cells with okadaic acid and observed increased mRNA of δ-aminolevulinate synthase and CYP2H1 whereas expression of CYP3A37 was decreased. The effects of okadaic acid and other modifiers of protein phosphorylation in LMH cells are comparable to those seen on CYP2Bs and CYP3As in mammalian primary hepatocyte cultures. These results indicate that closely related nuclear receptors, transcription factors, and signaling pathways are mediating the transcriptional activation of multiple genes by xenobiotics in chicken, rodents, and man. (*Molecular Endocrinology* 15: 1571–1585, 2001)

A GENE SUPERFAMILY of heme proteins, the cytochromes P-450 (CYP), encodes for the main enzymatic system for metabolism of lipophilic compounds of diverse structures (1). A common feature of most of these CYP substrates, which include drugs, fatty acids, cholesterol precursors, and metabolites such as steroid hormones or bile acids, is their hydrophobicity, which enables direct diffusion into cells and binding to CYPs as well as intracellular receptors (2–8). A subset of these CYPs can be induced or inhibited in the liver by a variety of substances, including their own substrates. Foreign compounds inducing CYPs can be categorized into different classes represented by the prototypical substrates dioxin, phenobarbital (PB), dexamethasone, clofibrate, and ethanol. For example, PB and PB-like inducers affect predominantly the CYP2B, CYP2C, and CYP3A subfamilies (2, 5–9). Apart from these CYPs, at least 50 other genes are influenced, triggering a pleiotropic hepatic reaction characterized by an increase in liver weight, proliferation of smooth endoplasmic reticulum, and tumor promotion as well as many other effects (10). PB induction of CYP has been described in a variety of species from *Bacillus megaterium* to man. Nevertheless, marked differences in induction or inhibition potentials of different drugs have been observed in different species. For instance, whereas the antiglucocorticoid 5-pregnen-3β-ol-20-one-16α-carbonitrile (PCN) is a strong inducer of CYP3As in rat and mouse, it barely changes CYP3A levels in man or rabbit (7, 11, 12). In contrast, 5β-pregnane-3,20-dione and RU486 (mifepristone)

Abbreviations: ALAS, δ-aminolevulinate synthase; CAR, constitutive androstane receptor; CAT, chloramphenicol acetyltransferase; CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; CXR, chicken xenobiotic receptor; CYP, cytochrome P-450; FXR, farnesoid X receptor; LMH, leghorn male hepatoma; PB, phenobarbital; PBRU, PB-responsive enhancer unit; PCN, 5-pregnen-3β-ol-20-one-16α-carbonitrile; PIA, propylisopropylacetamide; PP, protein phosphatase; PXR, pregnane X receptor; SSC, NaCl-sodium citrate; tk, thymidine kinase.

are potent CYP3A inducers in man and mouse but induce to a lesser extent in rabbit or rat (7, 11, 12).

Although the inducing effect of PB and other drugs was discovered more than 40 yr ago, progress in understanding the molecular mechanisms of induction was limited until recently, when transfectable primary hepatocyte cultures of mice, rat, and chicken were developed. Reporter gene assays in these *in vitro* systems allowed the characterization of drug-responsive elements in the flanking regions of several inducible genes and transactivation assays in conjunction with EMSAs identified transcription factors that bind to these elements (13–22). However, primary cultures of hepatocytes have significant drawbacks that hinder the elucidation of molecular details linking the drug-induced signal to the transcription machinery. For instance, in primary hepatocyte cultures, the response to PB is often delayed, attenuated, and terminated rapidly. In addition, cultured hepatocytes may express an abnormal CYP profile. Primary hepatocytes are difficult to transfect, and the time needed for transfection and drug treatment is excessively long. Moreover, hepatoma-derived cell lines such as HepG2 do not express a complete set of CYPs and have lost the response to PB (for reviews, see Refs. 23 and 24).

In search of a convenient culture system, we discovered the leghorn male hepatoma cell line (LMH) to respond to PB and PB-like inducers to the same extent as primary chicken hepatocytes or chick embryo liver *in ovo*. The LMH cell line was established by inducing a hepatocellular carcinoma with diethylnitrosamine in a male leghorn chicken (25). Characteristically, the LMH cells feature a well differentiated morphology and biochemistry (25, 26). The biochemical and chromosomal properties, a triploid karyotype with six marker chromosomes, remain constant over a prolonged period of propagation in culture (25).

Here, we describe the characterization of PB-type induction in the LMH cells using CYP2H1 and CYP3A37 as well as δ-aminolevulinate synthase (ALAS) as representative genes regulated by PB. CYP2H1 is one of the major PB-inducible enzymes in chicken liver (27). CYP3A37, the first avian CYP of the 3A subfamily, was discovered in our laboratory and shown to be PB inducible (28). ALAS is the first and rate-limiting enzyme of heme biosynthesis (29). It is activated to meet the increased need of heme for CYP heme-proteins (30).

The flanking region of one of these genes, chicken CYP2H1, has recently been analyzed in detail and a PB-responsive enhancer unit (PBRU) has been defined (17, 19). Interestingly, the arrangement of certain putative transcription factor recognition sites, in particular a nuclear receptor consensus sequence arranged as a direct repeat of two hexamers separated by four nucleotides (DR-4) in the vicinity of a nuclear factor-1 site on the chicken PBRU, is highly conserved in comparison to PBRUs of mammalian PB-inducible CYPs (19, 31). This avian 264-bp enhancer element could be activated by the recently discovered chicken drug-sensing orphan nuclear receptor, chicken xenobiotic receptor (CXR) (22). Sequence comparisons revealed that CXR is closely related to both mammalian xenobiotic-activated receptors [constitutive androstane receptor (CAR) and pregnane X receptor (PXR)] showing between 61% and 67% amino acid identity in the DNA-binding domains and between 49% and 56% amino acid identity in the ligand-binding domains, respectively (22). We therefore wanted to test whether these orphan nuclear receptors interact with the avian PBRU.

Both drug-induction and orphan nuclear receptor signaling have been reported to be highly influenced by phosphorylation and dephosphorylation events (32–35). We studied the effects of the protein phosphatase inhibitor okadaic acid and the cAMP modulator forskolin on CYP2H1, CYP3A37, and ALAS as well as the effects of inducers on cAMP levels in the LMH cells to determine whether phosphorylation and dephosphorylation events elicit the same effects in LMH cells as those previously reported for primary cultures of rat and mouse hepatocytes (36–40).

The data presented here demonstrate the interchangeability of drug-responsive elements and xenobiotic-activated nuclear receptors in chicken and mammals and also suggest conservation of protein phosphorylation and dephosphorylation effects on induction in these different species.

RESULTS

PB Induction of CYP2H1 in the Chicken Hepatoma Cell Line LMH

To assess the inducibility of the LMH cell line by xenobiotics, CYP2H1 transcript and protein levels were measured after drug treatment. Induction of CYP2H1 mRNA was dose dependent with maximal transcript levels after treatment with 1 mM PB as shown by both semiquantitative PCR and Northern blot (Fig. 1A). Higher PB concentrations produced significant cell toxicity. In Northern blots, a band of about 3.5 kb corresponding to CYP2H1 was observed (Fig. 1A). As shown in Fig. 1B, transcript levels of CYP2H1 continued to rise even after 30 h of exposure to 600 μM PB. PB induction of CYP2H1 was dose dependent and reversible at the mRNA level. Similar results were obtained with mRNA of δ-aminolevulinic acid synthase (data not shown) and CYP3A37 (28). When comparing protein levels of CYP2H1 in chicken embryo liver (*in ovo*) with those of chicken primary hepatocytes and LMH cells, we observed that control and PB-treated protein levels in the LMH cells reflected the *in ovo* situation better than the chicken primary hepatocytes (Fig. 1C). LMH cells were exposed to 600 μM PB for 24 h, chicken embryo hepatocytes were exposed to 1.2 mM PB for 48 h, and chick embryos were treated with 3 mg PB per egg for 48 h. Basal levels *in ovo* and

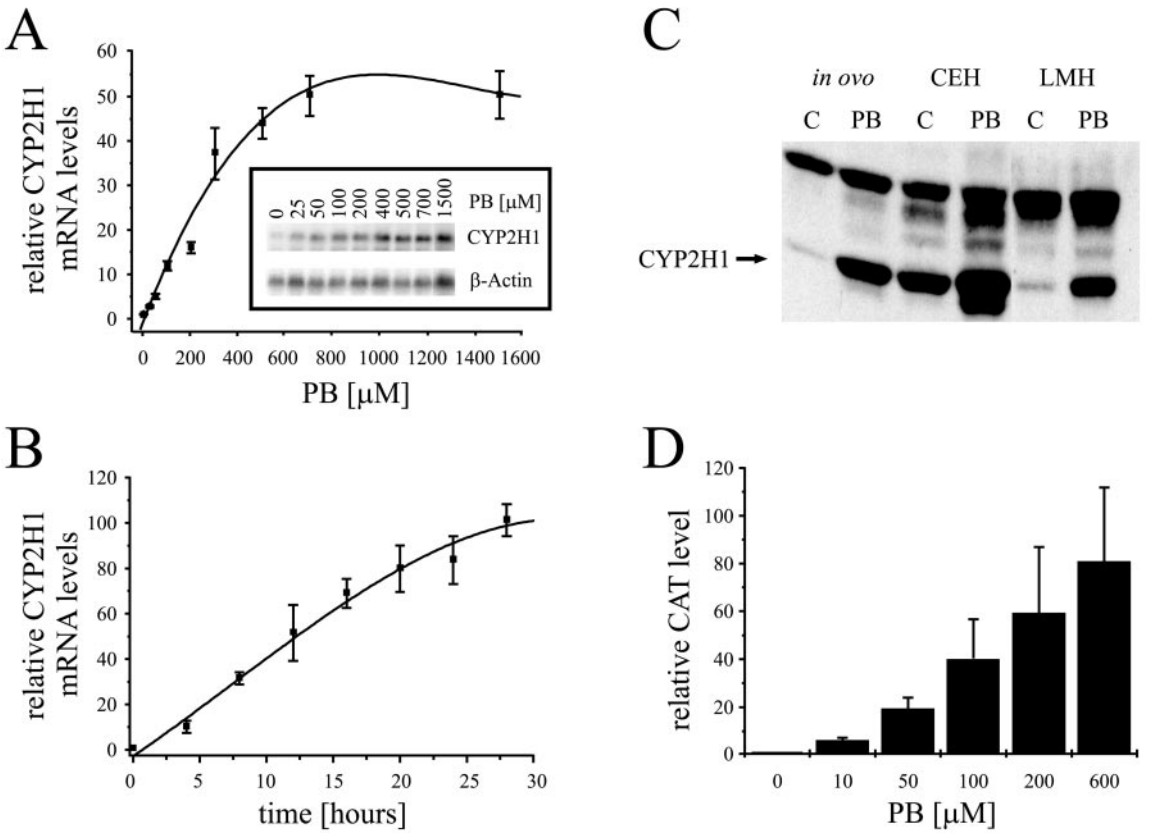

Fig. 1. Inducibility of LMH Cells

A, LMH cells were exposed to different concentrations of PB for 24 h. RNA was isolated and reverse-transcribed, and the level of CYP2H1 and β-actin cDNA was measured by semiquantitative PCR and Northern blot. CYP2H1 levels were adjusted relative to β-actin levels. Data represent the average of three independent experiments with *error bars* representing standard deviations. B, LMH cells were treated with 600 μM PB for the indicated time period, and transcript levels of CYP2H1 and β-actin were measured. Data represent the average of three independent experiments with *error bars* representing standard deviations. C, LMH cells were exposed to 600 μM PB for 24 h, chicken embryo hepatocytes (CEH) were exposed to 1.2 mM PB for 48 h, and chicken embryos *in ovo* were treated with 3 mg PB per egg for 48 h before preparation of liver homogenate. Protein from chicken liver homogenate, primary cultures of chicken hepatocytes, and LMH cells was isolated and a Western blot performed with CYP2H1 antibodies as described in *Materials and Methods*. D, LMH cells were transfected with the 4.8-kb PB-responsive enhancer from CYP2H1 in pBLCAT5 containing an enhancerless tk-promoter. After exposure to the indicated concentrations of PB for 48 h, a CAT ELISA was performed. The relative CAT expression was standardized against untreated control cells and expressed as fold induction. Data represent the average of three independent experiments with *error bars* representing standard deviations.

in the LMH cells were lower than those in chicken embryo hepatocytes, but consistent induction could be seen in all three systems. The identities of the upper bands in the Western blot are not yet clear. We have found members of the CYP2C subfamily in chicken (data not shown), and these bands might represent CYP2Cs that are recognized by the polyclonal CYP2H1 antibody due to the close relationship of CYP2Cs and CYP2Hs. Nevertheless, these bands are clearly distinguishable from the band representing CYP2H1 by their higher molecular weight. Induction of CYP3A37 protein by PB in the LMH cells has previously been demonstrated (28).

A 4.8-kb fragment of the 5′-flanking region of CYP2H1, which has been shown to be a PB-responsive enhancer element (41), was cloned into a pBLCAT5-reporter vector containing an enhancerless thymidine kinase (tk)-promoter and transfected into LMH cells. Nonradioactive chloramphenicol acetyltransferase (CAT) reporter gene assays were performed, and a dose-dependent increase in CAT protein was observed amounting to a 60-fold induction at 200 μM PB after 48 h (Fig. 1D). This response is higher than the induction levels obtained in chicken primary hepatocytes after a 48 h exposure to 400 μM PB, resulting in a 20-fold increase (data not shown). In experiments reported by another laboratory (17, 41), the 4.8-kb enhancer fragment cloned into a pCAT-reporter vector containing the weak, enhancerless SV40 promoter gave a 7- to 14-fold induction after treatment of chicken primary hepatocytes with 500 μM PB for 48 h. Thus, LMH cells provide a reliable tool that is as highly drug-responsive as are *in ovo* systems (Fig. 1C).

Mammalian Drug-Responsive Enhancer Elements Are Activated in the Chicken LMH Cells

In previous experiments, we have observed that a 51-bp PBREM from the mouse Cyp2b10 5′-flanking region (15) was activated by PB-like inducers in the LMH cell line (19). To extend these studies, we tested the response elements of the rat CYP2B2 (13, 16), the human CYP2B6 (18), and the mouse Cyp2b10 (15) gene with different inducers in LMH cells and compared the results to the findings of the chicken 264-bp PBRU (19). The 163-bp PBRU of rat CYP2B2, the 51-bp PBREM of human CYP2B6, the 51-bp PBREM of mouse Cyp2b10, and the 264-bp PBRU of chicken CYP2H1 subcloned in reporter gene vectors were transfected into LMH cells, and reporter gene assays were performed after applying different inducers for 16 h (Fig. 2). We selected the same drugs as those used in previous studies of the chicken 264-bp PBRU and the mouse Cyp2b10 PBREM (19). As prototypical CYP2B gene activators, we used PB (400 μM) and the PB-like inducers propylisopropylacetamide (PIA) (250 μM) and glutethimide (500 μM). The two CYP3A inducers dexamethasone (50 μM) and metyrapone (400 μM) were also examined. Rifampicin (100 μM) and PCN (50 μM) have species-specific effects on CYP3As and were therefore of interest. Furthermore, LMH cells were treated with the prototypical CYP1A1-inducer β-naphtoflavone (10 μM). In general, both the human and the rat response element reacted in the same way to the different inducers varying only in the extent of the inductions (Fig. 2, A and B). PB-induction of the rat 163-bp PBRU was 6-fold in contrast to the human 51-bp PBREM that was 1.5-fold induced. High induction on both elements was observed when using PIA, glutethimide, or metyrapone. PIA conferred a 68-fold induction on the rat PBRU and a 6.1-fold induction on the human PBREM, slightly higher than the 34-fold induction of the rat PBRU and the 4.2-fold induction of the human PBREM by glutethimide. Metyrapone induced the rat PBRU 46-fold and the human PBREM 8.1-fold. Dexamethasone, PCN, rifampicin, and β-naphtoflavone had no or only very minor effects on both elements (Fig. 2, A and B). These results demonstrate that the rat 163-bp PBRU and the human 51-bp PBREM react comparably to the chemicals. Moreover, these inductions correspond to the reporter gene activations by the chicken 264-bp PBRU and the mouse 51-bp PBREM in LMH cells (Fig. 2, C and D). The different relative magnitude of the responses to different drugs might be explained by recent findings demonstrating that the 163-bp PBRUs and the 51-bp PBREMs are not equivalent in their response to different inducers and that additional transcription factor-binding sites on the 163-bp PBRU contribute to maximal induction (42). The mouse and the chicken enhancer reacted almost identically to drug treatment as observed previously (19). Consequently, the same or very similar receptors in the chicken LMH cells can bind to and activate the different response elements from human, rat, mouse, and chicken after drug treatment.

Human PXR and Human CAR Bind to the Chicken CYP2H1 264-bp PBRU in Gel-EMSAs

Since the drug-response elements are exchangeable between chicken, mouse, rat, and human, we wanted to test whether the different xenobiotic-sensing nuclear receptors PXR, CAR, and CXR from human and chicken bind to the CYP2H1 264-bp PBRU. None of the *in vitro* transcribed/translated receptors bound to radiolabeled 264-bp PBRU as monomers (Fig. 3, lanes 2–5). Heterodimerized with chicken RXRγ, both human PXR and human CAR formed a complex on the CYP2H1 PBRU as did the CXR/RXRγ complex (Fig. 3, lanes 6, 8, and 10, region b). These complexes could be supershifted when using an antibody against RXR (Fig. 3, lanes 7, 9, and 11, region c). Thus, all three drug-activated nuclear receptors, PXR, CAR, and CXR, could be shown to interact with the chicken PBRU.

Human and Mouse PXR Transactivate the Chicken 264-bp PBRU in CV-1 Cells

As mammalian xenobiotic-sensing orphan nuclear receptors are binding to the chicken 264-bp PBRU, functional tests were performed to determine whether these receptors also activate the avian response element. In contrast to CXR and PXR, CAR activation by PB is not measurable in CV-1 cell transactivation assays for the following reasons: 1) CAR is a constitutively active transcription factor (43, 44); 2) PB apparently is not a ligand of CAR but induces by an indirect mechanism involving cytoplasmic-nuclear transfer of CAR (35, 45); 3) transiently or stably expressed CAR is exclusively nuclear in cultured cells (35, 45). Due to these limitations, we restricted the transactivation experiments to PXR and CXR in this study. Monkey kidney epithelial CV-1 cells were cotransfected with the reporter gene construct containing the 264-bp PBRU and the expression plasmid for CXR, human PXR, and mouse PXR, respectively. Cells were treated for 24 h with different inducer compounds and reporter gene levels were measured. Mammalian PXRs and chicken CXR all were able to transactivate the chicken 264-bp PBRU. Moreover, activation patterns corresponded to the relative potencies of induction observed with a CYP3A4-responsive enhancer module and recapitulated the typical species-specific differences between human and mouse PXR (46). Thus, in CV-1 cells, human PXR was strongly activated by PB, glutethimide, metyrapone, RU486, rifampicin, and clotrimazole whereas dexamethasone, RU486, and PCN activated mouse PXR (Fig. 4, A and B). The CYP2H1 264-bp PBRU is also activated by the chicken drug-activated orphan nuclear receptor CXR that was recently discovered in this laboratory (22). When comparing the activation patterns of mouse PXR, human PXR, and chicken CXR, CXR had almost identical

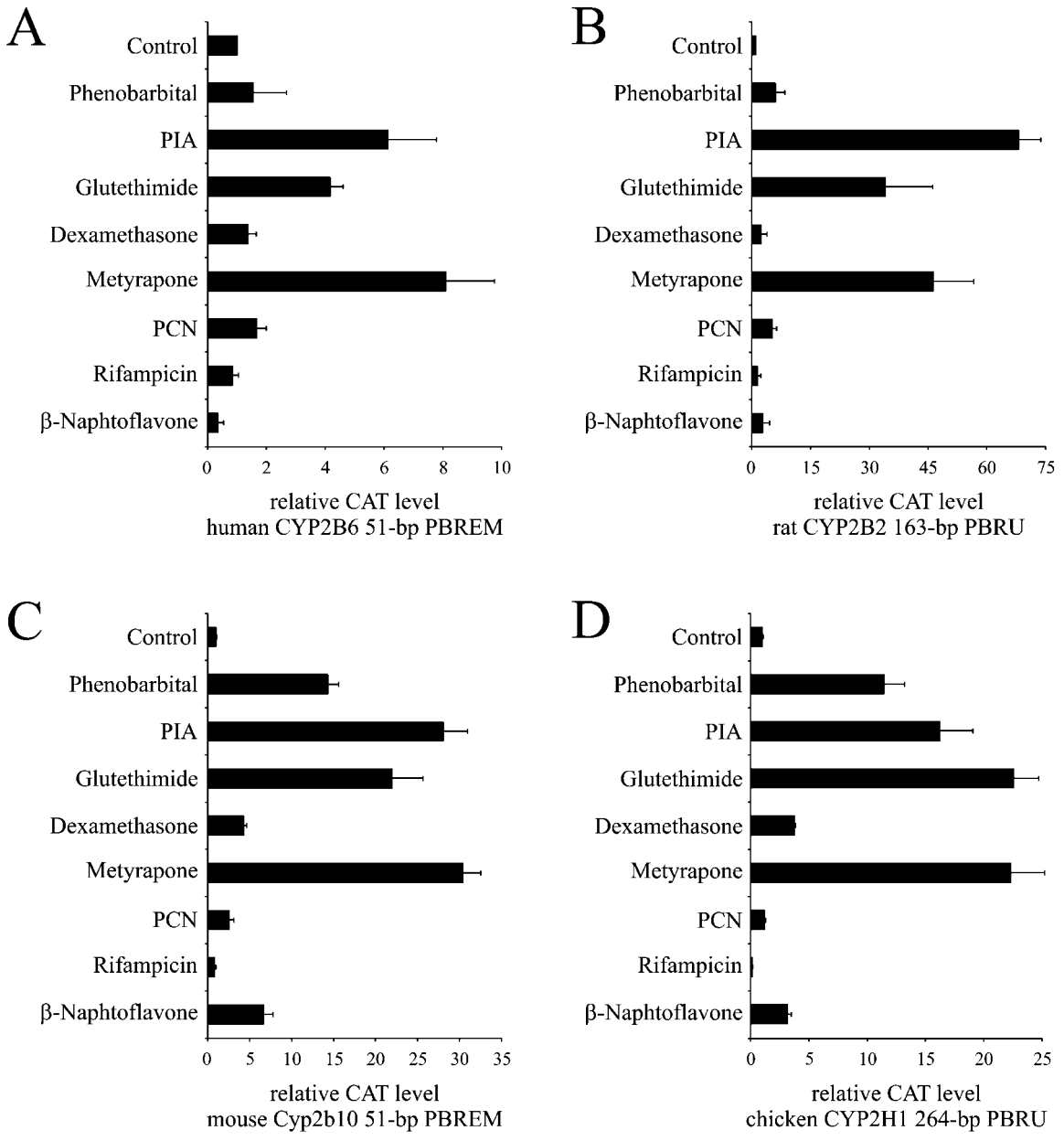

Fig. 2. Trans-species Activation of Rat CYP2B2 163-bp PBRU, Human CYP2B6 51-bp PBREM, Mouse Cyp2b10 51-bp PBREM, and Chicken CYP2H1 264-bp PBRU in LMH Cells

The PB-responsive elements from human CYP2B6 (A), rat CYP2B2 (B), mouse Cyp2b10 (C), and chicken CYP2H1 (D) genes were cloned into the pBLCAT5 reporter vector containing a tk promoter as described in *Materials and Methods* and Ref. 19. These constructs were transfected into LMH cells in suspension, and the cells were induced for 16 h with PB (400 μM), PIA (250 μM), glutethimide (500 μM), dexamethasone (50 μM), metyrapone (400 μM), PCN (50 μM) rifampicin (100 μM), or β-naphtoflavone (10 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 with *error bars* representing standard deviations.

specificity for ligands as human PXR concerning PB, dexamethasone, metyrapone, PCN, clotrimazole, and TCPOBOP (Fig. 4, A and C). Overlap between CXR and mouse PXR activation patterns was also observed after treatment with glutethimide and rifampicin (Fig. 4, B and C). In contrast, CXR was the only receptor that was activated by β-naphtoflavone and by PIA and the only receptor not affected by RU486 (Fig. 4C). These data indicate overlapping ligand specificity for these evolutionary distant receptors. Moreover, all three receptors could bind to the chicken enhancer element and transactivate this PBRU upon drug treatment using the transcriptional machinery of the monkey epithelial kidney cells.

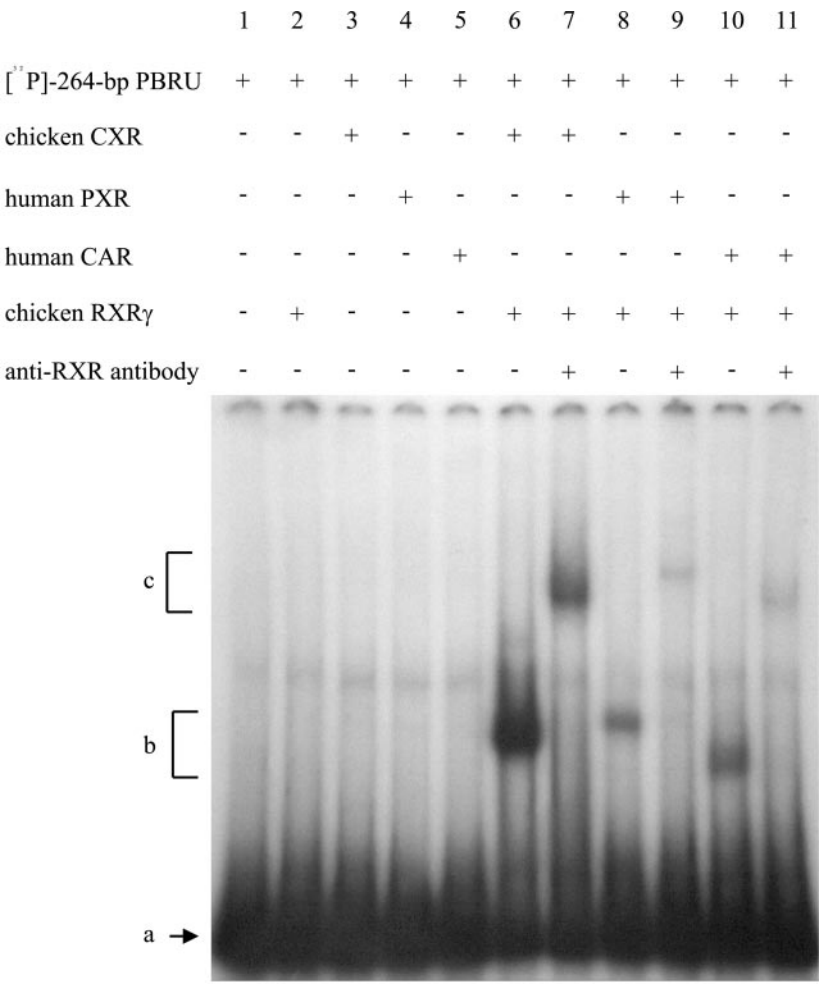

Fig. 3. Human PXR and Human CAR Bind to the Chicken 264-bp PBRU in EMSAs

Radiolabeled 264-bp PBRU was incubated with *in vitro* transcribed/translated CXR (lanes 3, 6, and 7), human PXR (lanes 4, 8, and 9), human CAR (lanes 5, 10, and 11), chicken RXRγ (lanes 2 and 6–11), and anti-RXR antibody (lanes 7, 9, and 11). The *arrow* depicts unbound probe (a). Complexes of CXR, human PXR, and human CAR with chicken RXRγ result in shifts (b) and with addition of anti-RXR antibody in supershifts (c).

The DR-4 Element Within the CYP2H1 264-bp PBRU is the Main Binding and Activation Site for PXR, CAR, and CXR

Nuclear receptor binding sites consisting of two direct repeats of nucleotide hexamers with the consensus sequence AGT/GTCA separated by four nucleotides (DR-4) have been identified to constitute the binding sites for xenobiotic-sensing orphan nuclear receptors on mammalian and chicken PBRUs and to confer drug inducibility of those (15, 16, 18, 19, 21). To assess the importance of this DR-4 in cross-species experiments, we compared the effects of the CYP2H1 264-bp PBRU containing mutated hexamer half-sites in its DR-4 (called "double," described in Ref. 19) to those of wild-type CYP2H1 264-bp PBRU (wt). EMSAs revealed that the mutated CYP2H1 264-bp PBRU was no longer able to bind either CXR, human PXR, or human CAR heterodimerized to chicken RXRγ (Fig. 5A, lanes 4, 6, and 8) in contrast to the wild-type 264-bp PBRU (Fig. 5A, lanes 3, 5, and 7). Moreover, the mutated 264-bp PBRU was also compared with its wild-type counterpart in CV-1 cell transactivation assays with human PXR, mouse PXR, and CXR. After transfection, CV-1 cells were treated with either vehicle [0.1% dimethylsulfoxide (DMSO)], RU486 (10 μM), PCN (50 μM), or clotrimazole (10 μM) for 24 h. Cell extracts were analyzed for CAT expression normalized against β-galactosidase levels. These compounds were chosen because of their ability to strongly induce either human PXR (RU486, clotrimazole), mouse PXR (RU486, PCN), or CXR (clotrimazole) as shown in Fig. 4. Activation levels of all three receptors were severely reduced on the 264-bp PBRU DR-4 double mutant compared with the wild-type PBRU (Fig. 5, B, C, and D). The species-specific induction levels of human PXR by RU486 and clotrimazole, mouse PXR by RU486 and PCN, and CXR by clotrimazole were strongly reduced on the 264-bp CYP2H1 PBRU DR-4

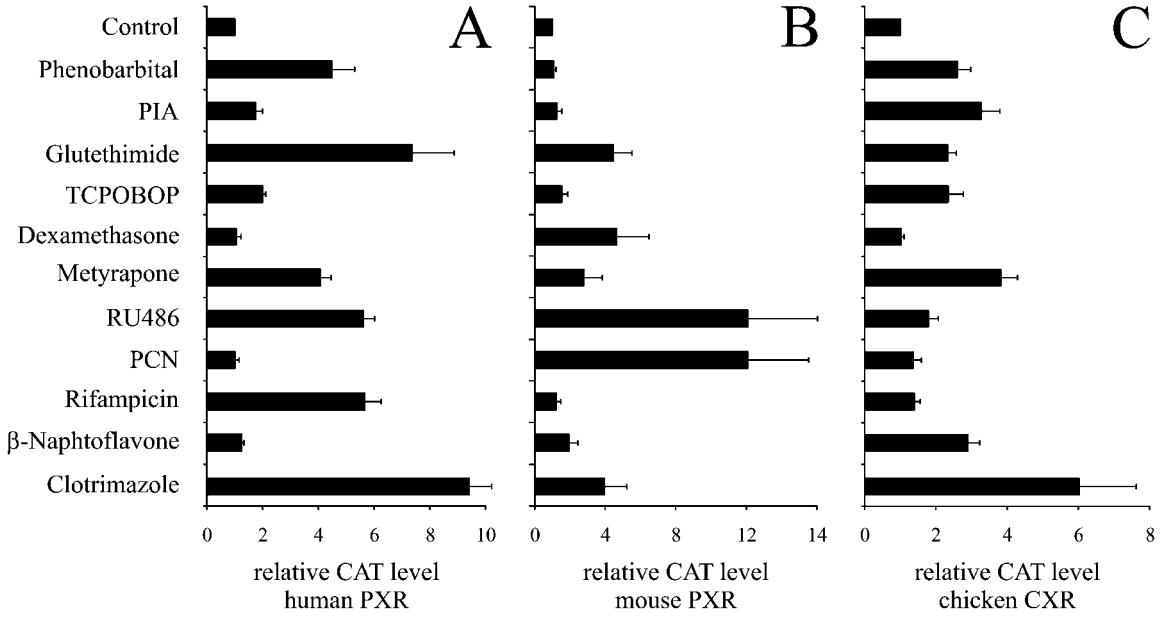

Fig. 4. Human and Mouse PXR and Chicken CXR Activate the Avian 264-bp PBRU in CV-1 Transactivation Assays

CV-1 cells were cotransfected with expression plasmids for either human PXR (A), mouse PXR (B), or chicken CXR (C) together with a CAT reporter gene plasmid containing the CYP2H1 264-bp PBRU as described in Ref. 22. Cells were then treated with vehicle (0.1% DMSO), PB (400 μM), PIA (250 μM), glutethimide (500 μM), TCPOBOP (10 μM), dexamethasone (50 μM), metyrapone (400 μM), RU486 (10 μM), PCN (50 μM), rifampicin (100 μM), β-naphtoflavone (10 μM), or clotrimazole (10 μM) for 24 h. Cell extracts were analyzed for CAT expression normalized against β-galactosidase levels. Values are the average of three independent experiments and the *error bars* represent standard deviations.

double mutant in comparison with the wild-type 264-bp PBRU. Both the EMSA and the transactivation experiments thus underline the importance of the DR-4 element within the CYP2H1 264-bp PBRU in drug induction.

Okadaic Acid Affects Drug-Induction of CYP2H1, CYP3A37, and ALAS

In diverse *in vitro* systems including rat and mouse primary hepatocyte cultures, both nuclear receptor activity and drug induction of CYPs have been shown to be modulated by diverse protein kinases and protein phosphatases (for reviews, see Refs. 32 and 34). Okadaic acid (OA) is an inhibitor of various protein phosphatases (PP) of which PP-1, PP-2A, and PP-2B are the best studied among those thought to be affected (47). We used OA as a tool to inhibit PPs and measure their effect on induction of ALAS, CYP2H1, and CYP3A37 by semiquantitative RT-PCR using the Taqman system. In preliminary dose-response experiments, OA induced CYP2H1 and ALAS mRNA levels at concentrations of about 100 nM whereas CYP3A37 mRNA levels were inhibited by 10 nM OA (data not shown). We therefore used 1 μM OA in the following coincubation experiments to obtain clear results for all three genes. To get a general overview of induction pathways affected by PP, we tested the effect of OA treatment on inducers of three different types, namely PB, dexamethasone, a synthetic glucocorticoid, and metyrapone, a substituted pyridine. LMH cells were induced with 400 μM PB, 50 μM dexamethasone, and 400 μM metyrapone alone and together with 1 μM OA or 1 μM 1-nor-okadaone, an inactive analog of OA. After 16 h, transcript levels of ALAS, CYP2H1, and CYP3A37 were determined (Fig. 6). PB, dexamethasone, and metyrapone induced ALAS, CYP2H1, and CYP3A37 with metyrapone being the strongest inducer, most impressive in the case of CYP3A37 (Fig. 6C). In contrast to 1-nor-okadaone, OA had profound effects on all three enzymes, *i.e*. increase of ALAS and CYP2H1 transcript level in contrast to complete inhibition of CYP3A37 induction by PB, dexamethasone, or metyrapone (Fig. 6). When OA was added in combination with PB, dexamethasone, or metyrapone, there was no increase in transcript levels of ALAS and CYP2H1 over the levels achieved with OA alone, suggesting that OA affects the mechanism by which drugs activate transcription (Fig. 6). This effect is best seen in coexposures to OA and metyrapone for ALAS, where transcript levels were even lowered by combined treatment compared with metyrapone alone. The effects of OA were confirmed at the protein level by Western blotting for CYP2H1 and CYP3A37 (data not shown). Thus, the inhibition of PB induction of CYPs, as observed for rat CYP2B2 and mouse Cyp2b10 in primary cultures of hepatocytes, could also be shown for chicken CYP3A37 on mRNA levels in LMH cells. The apparently paradoxical CYP2H1 mRNA induction

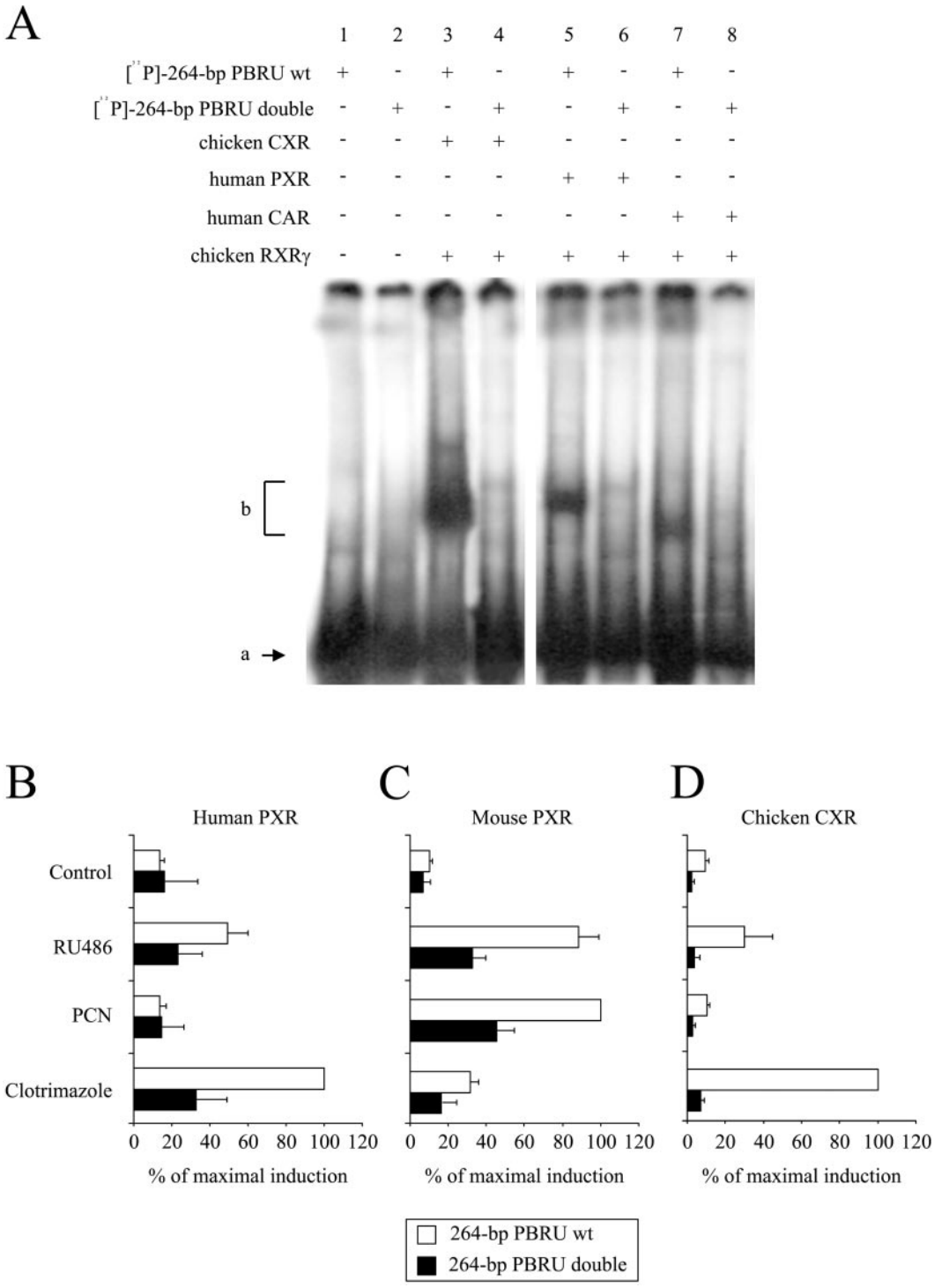

Fig. 5. The DR-4 Element Within the CYP2H1 264-bp PBRU Confers Binding and Inducibility by PXR, CAR, and CXR

A, Radiolabeled wild-type 264-bp PBRU (wt, lanes 1, 3, 5, and 7) or 264-bp PBRU mutated in both hexamers of the DR-4 element (double, lanes 2, 4, 6, and 8) were incubated with *in vitro* transcribed/translated CXR (lanes 3 and 4), human PXR (lanes 5 and 6), and human CAR (lanes 7 and 8) together with chicken RXRγ (lanes 3–8). The *arrow* depicts unbound probe (a) and the complexes of CXR, human PXR, and human CAR with chicken RXRγ resulting in a shifts (b). B, C, and D, CV-1 cells were cotransfected with expression plasmids for either human PXR (B), mouse PXR (C) or chicken CXR (D) together with a CAT reporter gene plasmid containing the wild-type CYP2H1 264-bp PBRU (wt) or the CYP2H1 264-bp PBRU containing mutated hexamer half-sites in its DR-4 element (double). Cells were then treated with vehicle (0.1% DMSO), RU486 (10 μM), PCN (50 μM), or clotrimazole (10 μM) for 24 h. Cell extracts were analyzed for CAT expression normalized against β-galactosidase levels. Values are the average of three independent experiments, expressed as percent of maximal induction levels, and the error bars represent standard deviations.

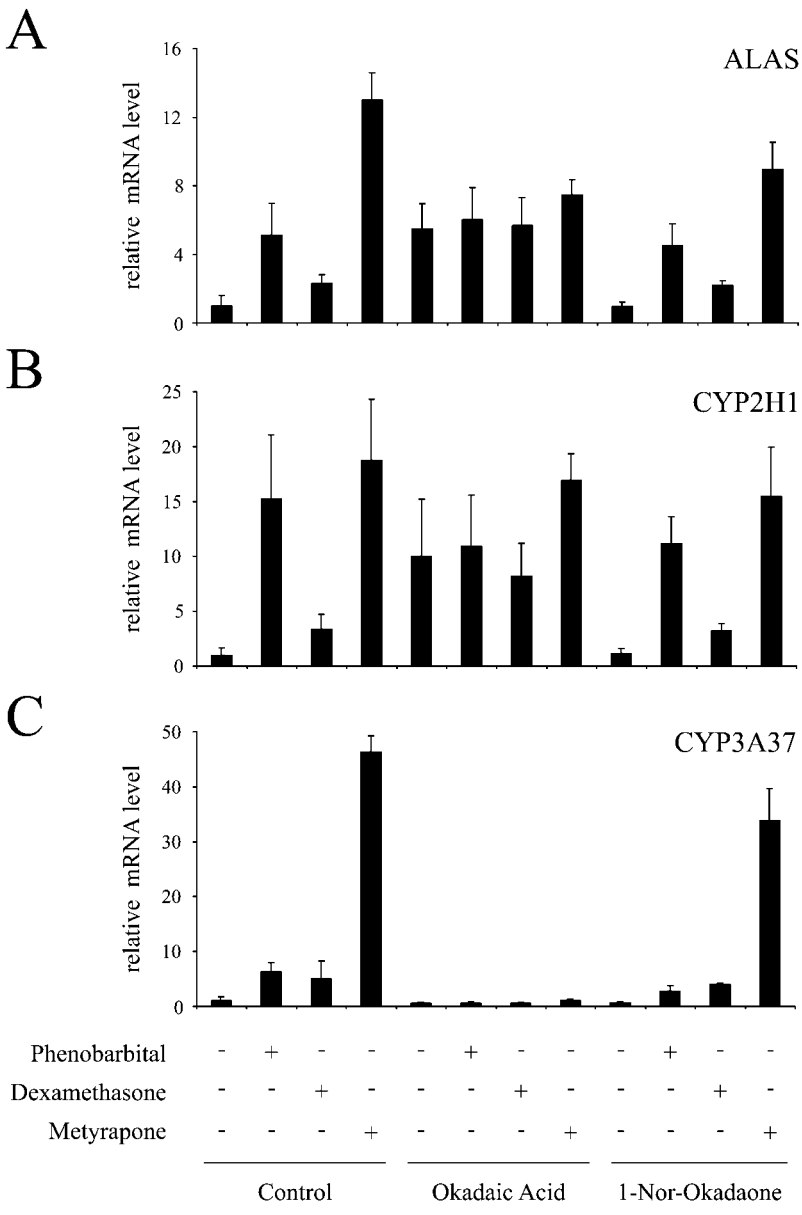

Fig. 6. OA Affects PB-, Dexamethasone-, and Metyrapone Induction of ALAS, CYP2H1, and CYP3A37

LMH cells were treated with 1 μM OA or 1 μM 1-nor-okadaone for 16 h in combination with either 400 μM PB, 50 μM dexamethasone, or 400 μM metyrapone. Total RNA was isolated and reverse transcribed. Relative mRNA levels of ALAS (A), CYP2H1 (B), and CYP3A37 (C) from treated cells against control cells were determined with a Taqman ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland) and normalized against GAPDH mRNA level as described in *Materials and Methods*. Values are the average of three independent experiments and the *error bars* represent standard deviations.

can be explained by previous findings showing that PB induction on the CYP2H1 264-bp PBRU is inhibited by OA and the observed overall induction of CYP2H1 is due to activation of its promoter by OA (19).

PB Does Not Change cAMP Levels in LMH Cells

To study drug-mediated changes in second messenger systems involved in phosphorylation and dephosphorylation events, we measured the intracellular cAMP levels in the LMH cells after treatment with PB and modulators of cellular cAMP levels using a nonradioactive, competitive immunoassay. In rat and mouse primary hepatocytes, PB modulated neither cAMP levels nor PKA activities (39, 40). In LMH cells, forskolin, an activator of adenylate cyclase, elevated intracellular cAMP 30 min after addition to the LMH cells from 0.9 pmol/ml to 1,323 pmol/ml (Fig. 7). Sixteen hours later, cAMP levels were not higher than in control cells, comparable to the kinetics described

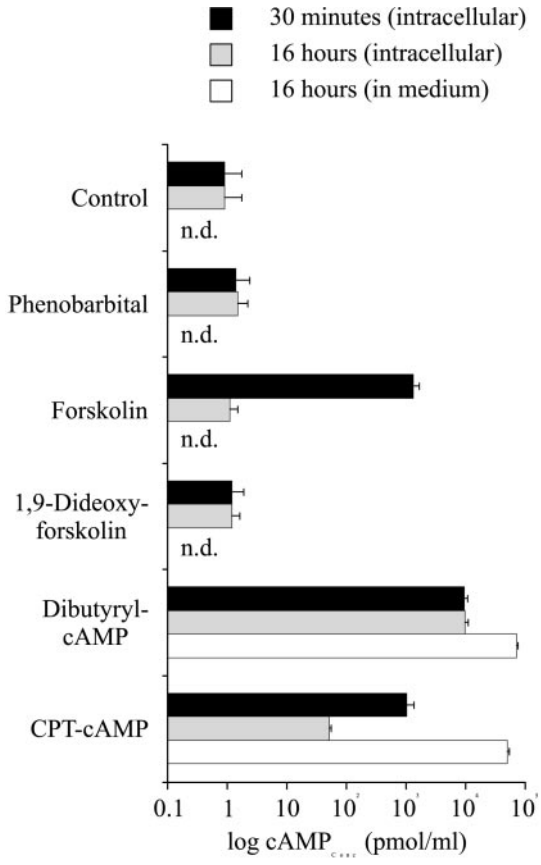

Fig. 7. Effect of Drug Treatment on cAMP Levels in the LMH Cells

LMH cells were treated with 400 μM PB, 100 μM forskolin, 100 μM 1,9-dideoxyforskolin, 100 μM $(Bu)_2$cAMP or 100 μM CPT-cAMP for either 30 min or 16 h. Cells were harvested, and a competitive immunoassay was used to measure intracellular cAMP and cAMP in the medium. Values are the average of three independent experiments and the *error bars* represent standard deviations. Concentrations below detection limits are designated n.d. (nondetectable).

previously in primary rat and mouse hepatocytes (36, 39, 40). Treatment with PB or 1,9-dideoxyforskolin, a negative analog of forskolin, had no effect on intracellular cAMP level. As expected, none of these three compounds could raise cAMP levels in the medium to a detectable concentration. The cAMP analog $(Bu)_2$cAMP could be detected in the cells, both after 30 min and after 16 h, at a high concentration of approximately 9,500 pmol/ml whereas another cAMP analog, 8-(4-chlorophenylthio)-cAMP (CPT-cAMP), was less potent (Fig. 7). Both analogs were designed for easy penetration in cell culture systems, and $(Bu)_2$cAMP was apparently more stable compared with CPT-cAMP in LMH cell cultures. These and previous results demonstrate that PB treatment of chicken LMH cells and of primary hepatocytes of rat and mouse has no effect on the second messenger cAMP.

Forskolin Activates CYP2H1, CYP3A37, and ALAS Independently of Adenylate Cyclase and cAMP

No effect of forskolin on CYP2b10 was observed in primary cultures of mouse hepatocytes (39), but forskolin affected rat CYP2B1/2 and rat CYP3A1 transcription in primary hepatocytes in different ways. In contrast to the inhibition of CYP2B1/2 (48), an induction of CYP3A1 by forskolin was proposed to be due to adenylate cyclase-independent effects since the analog 1,9-dideoxyforskolin, which shows no adenylate cyclase-activation, had the same inducing effect (36). Transcript levels of CYP2H1, CYP3A37, and ALAS were determined after treating LMH cells for 16 h with 400 μM PB, 100 μM forskolin, 100 μM 1,9-dideoxyforskolin, or 100 μM $(Bu)_2$cAMP. No effect of $(Bu)_2$cAMP on any of the three enzymes was observed (Fig. 8) whereas both forskolin and 1,9-dideoxyforskolin elevated mRNA levels of ALAS, CYP2H1, and CYP3A37 (Fig. 8). Thus, the effect of forskolin and 1,9-dideoxyforskolin is apparently independent of adenylate cyclase activation and cAMP levels in the chicken LMH cells, similar to regulation of rat CYP3A1 in primary hepatocyte cultures (36). Whereas CYP2B2 and CYP3A1 in rat both were inhibited by cAMP (50), our results correlate with the lack of effect of cAMP on mouse Cyp2b10 in primary hepatocytes (39) showing no effect of cAMP on PB-inducible enzymes. Thus, comparisons of our data with previous results in primary cultures of rat and mouse hepatocytes show a high conservation of protein phosphorylation and dephosphorylation events in enzyme induction triggered by drug treatment between chicken and rodents (36, 37, 39, 40).

DISCUSSION

Drug-Responsive Enhancer Units and Xenobiotic-Sensing Receptors Are Conserved in Chicken, Rodents, and Man

CYP induction by drugs and xenobiotics has been observed in a wide range of species (2, 4–9). However, there are major interspecies differences in the spectrum of inducer drugs and the pattern of activated genes (7, 11, 12). The molecular origin of this divergence has remained unclear. The results summarized in this report demonstrate conservation of the basic mechanism of drug induction between different species and suggest that divergent ligand-binding domains of orphan nuclear receptors account for the observed species differences. These conclusions are deduced from the following observations: First, drug-responsive elements from the human CYP2B6, the rat CYP2B2, the mouse Cyp2b10, and the chicken CYP2H1 could be activated in the chicken LMH cells. The response elements showed similar activation patterns after treatment with different drugs that closely resembled the CXR-mediated activation pattern of the

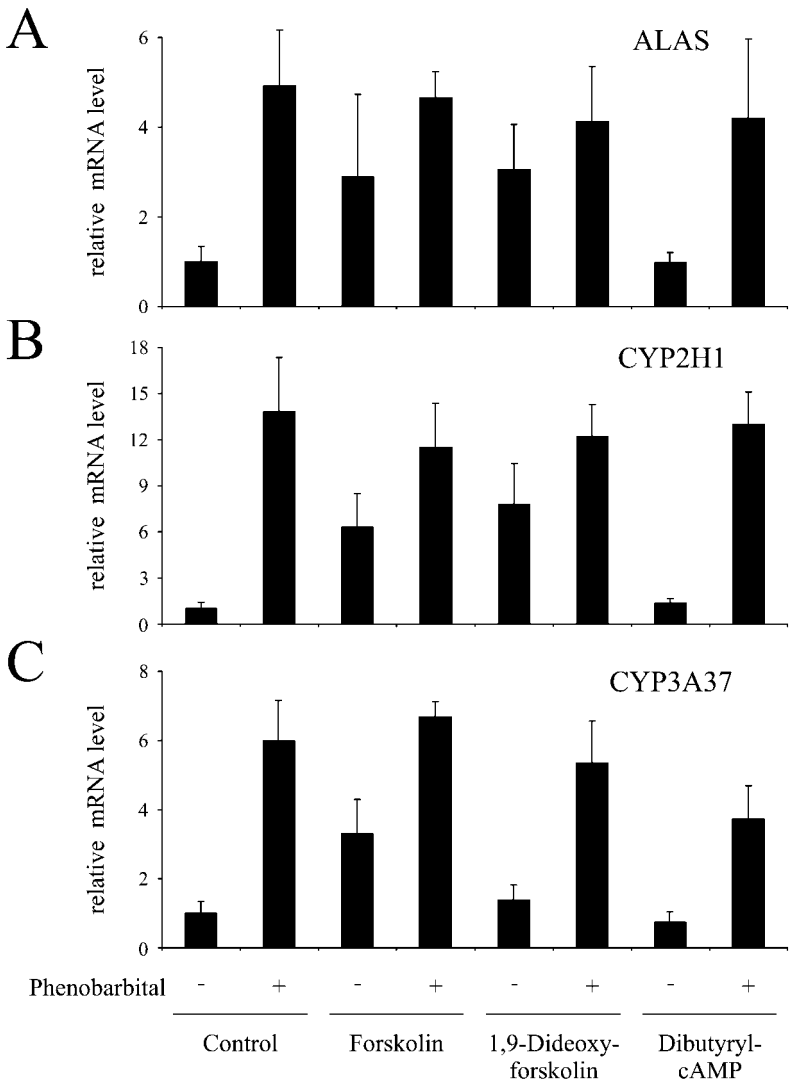

Fig. 8. Influence of Forskolin and cAMP on ALAS, CYP2H1, and CYP3A37

LMH cells were treated with 100 μM forskolin, 100 μM 1,9-dideoxyforskolin, or 100 μM $(Bu)_2$cAMP for 16 h with or without 400 μM PB. Total RNA was isolated and reverse transcribed. Relative mRNA levels of ALAS (A), CYP2H1 (B), and CYP3A37 (C) from treated cells against control cells were determined with a Taqman ABI PRISM 7700 Sequence Detection System and normalized against GAPDH mRNA level as described in *Materials and Methods*. Values are the average of three independent experiments and the *error bars* represent standard deviations.

chicken CYP2H1 PBRU in CV-1 cell transactivation assays as well. These data suggest that mammalian enhancers also interact with CXR. Similarly, all elements are highly conserved in their structure of transcription factor binding sites of which a direct repeat of hexamer half-sites separated by four nucleotides (DR-4) is responsible for binding of the xenobiotic-sensing orphan nuclear receptor heterodimers (19). Second, the three different members of drug-sensing orphan nuclear receptors CXR, PXR, and CAR bound the chicken 264-bp PBRU in EMSAs. Moreover, in CV-1 cell transactivation assays, the mammalian PXRs could activate the chicken CYP2H1 PBRU to the same extent as the chicken CXR. Thus, in monkey CV-1 cells, the dynamic multiprotein complex required for the induction machinery is able to cross-react with the chicken, human, and mouse receptors and the chicken response element. We interpret these data as support for the concept that species differences in drug induction are due to divergent ligand-binding domains of the xenobiotic-sensing orphan nuclear receptors and not to fundamental differences in the induction mechanism. The 60% to 80% amino acid sequence similarity in the ligand-binding domain of drug-activated orphan nuclear receptor orthologs is significantly lower than the 90% amino acid similarity observed in principle between nuclear receptor orthologs. Accordingly, mice devoid of endogenous PXR that are rescued with human PXR exhibit a humanized xenobiotic response (49). These findings indicate that it is exclusively the ligand-binding domain of drug-sensing orphan nuclear receptors that independently

diverged during evolution of species as a reaction to different environmental challenges (7, 12, 49).

Drug-Induction Mechanisms in Birds and Mammals Are Similarly Affected by Protein Phosphorylation and Dephosphorylation Events

The conservation of xenobiotic-triggered signaling pathways and the protein-protein interactions involved therein are further supported by our observations on the effect of modifiers of protein phosphorylation on drug induction. Although only CYP3A37 mRNA levels were reduced by OA, we showed previously that induction of CYP2H1 by OA is predominantly mediated by a 205-bp core promoter fragment whereas drug induction of the 264-bp PBRU is abolished by OA (19). Thus, the inhibition of CYP induction by OA correlates with the CYP inhibitions in rat and mouse. Moreover, nuclear translocation of the drug-sensing orphan nuclear receptor CAR triggered by drug treatment could be inhibited by OA leading to repression of CYP induction in mouse liver (35). These results suggest that PPs play an important role in the molecular mechanism of CYP induction. In contrast, cAMP levels were not affected by drug treatment, and the CYP induction triggered by the adenylate-cyclase activator forskolin apparently is not linked to changes in cAMP in the cell. Forskolin mediates a multitude of physiological effects that cannot be reproduced by cAMP analogs, *i.e.* inhibiting a variety of membrane proteins such as membrane transporters, voltage-sensitive channels, or P-glycoprotein. The diterpenic structure of forskolin is related to steroids, and forskolin might therefore exhibit steroid-like properties (see Ref. 36 and references therein). More recently, forskolin was found to activate the farnesoid X receptor (FXR), the bile acid receptor in liver, kidney, and intestine that is involved in regulation of CYP7A, a key enzyme in cholesterol homeostasis (50–52). Overlapping ligand affinities to different orphan nuclear receptor also have been observed for troglitazone, a drug used for treatment of type 2 diabetes that activates both the PPAR and PXR. Similarly, SR12813, a compound that lowers cholesterol levels in a number of species, activates both FXR and PXR (12). These findings indicate that most, if not all, xenobiotic inducer compounds affect more than one nuclear receptor system and that pronounced cross-talk exists between receptors that bind lipophilic drugs, fatty acids, steroids, and cholesterol derivatives such as CXR, PXR, CAR, FXR, and PPAR. In summary, these results in LMH cells demonstrate that both the orphan nuclear receptors and the signaling mechanisms affecting drug-induction pathways are similar in different species in spite of previous findings that proposed different proteins and transcription factors to be responsible for CYP induction in chicken compared with mammals (17).

The studies reported in this manuscript were possible by using a new, drug-inducible experimental system, the chicken hepatoma cell line LMH. The LMH cells offer the advantages of a continuously dividing culture system, including the availability of frozen stocks of clonal origin, long-term culture, high comparability, and transfection efficiency. LMH cells therefore allow the development of new experimental tools to study the molecular details of the effects of xenobiotics on gene expression, such as subclones with permanently expressed or mutated transcription factors or induction-resistant cell mutants suitable for complementation experiments.

MATERIALS AND METHODS

Reagents

OA sodium salt, 1-nor-okadaone, forskolin, and 1,9-dideoxyforskolin were purchased from Alexis Biochemicals (Läufelfingen, Switzerland). Dexamethasone, metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone), PCN, rifampicin, clotrimazole (1-[o-chlorotrityl]-imidazole), CPT-cAMP, and $(Bu)_2$cAMP) were obtained from Sigma (Buchs, Switzerland). PIA was generously provided by Dr. P. Sinclair (VA Hospital, White River Junction, VT). Glutethimide and β-naphtoflavone were purchased from Aldrich (Buchs, Switzerland). RU486 was obtained from Roussel-UCLAF SA (Paris, France). TCPOBOP (1, 4-bis[2-(3, 5-dichloropyridyloxy)]benzene) was a gift from Dr. U. Schmidt (Institute of Toxicology, BAYER AG, Wuppertal, Germany). PB sodium salt (5-ethyl-5-phenylbarbituric acid sodium salt) was purchased from Fluka Chemical Co. (Buchs, Switzerland). All other reagents were from standard suppliers. Cell culture media, sera, and tissue culture reagents were purchased from Life Technologies, Inc. (Basel, Switzerland) unless noted otherwise.

Plasmids

The rat CYP2B2 163-bp PBRU was amplified from rat genomic DNA, and the PCR product was digested with *Sau*3AI and subcloned into a *Bam*HI-digested pBlueScript vector. From a clone containing two copies in head-to-tail orientation, the insert was excised with *Xba*I and *Hin*dIII and ligated into the pBLCAT5 reporter gene vector. The coding regions of human and mouse CAR were amplified from human and mouse cDNA, respectively, followed by subcloning into the expression vector pSG5 (Stratagene, Basel, Switzerland). The expression vectors for human and mouse PXR, pSG5-hPXR, and pSG5-mPXR.1, kindly provided by Dr. S. A. Kliewer (Department of Molecular Endocrinology, Glaxo Wellcome Inc. Research and Development, Research Triangle Park, NC), were described in previous publications (11, 20). The reporter gene vector containing the human CYP2B6 51-bp PB-responsive enhancer module (PBREM), a kind gift of Dr. M. Negishi (NIEHS, NIH, Research Triangle Park, NC), was described previously (18).

Culture and Transfection of LMH Cells

LMH cells were obtained from the American Type Culture Collection (Manassas, VA) and thawed immediately after arrival. Cultivation in William's E medium and transfection with FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals, Rotkreuz, Switzerland) were performed as described previously (22).

cAMP Determination

The levels of cAMP were determined by using the Format A Cyclic AMP Enzyme Immunoassay Kit (BIOMOL Research

Laboratories, Inc., Plymouth Meeting, PA) according to the instructions of the manufacturer. Briefly, cells were incubated with the drugs for 30 min or 16 h and lysed. Intracellular cAMP and cAMP levels in the medium were measured using a competitive immunoassay.

Analysis of Reporter Gene Expression

Sixteen hours 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 SQRT-PCR Analysis

RNA purification and cDNA synthesis were performed as described previously (30). The primers for each cDNA amplification were designed to be specific and selective for the predicted sequences. For chicken CYP2H1, the primers used for RT-PCR analysis were the following: forward 5′-GAC ACT TGA CAT CTC TTC CTC-3′, and reverse 5′-CTG GGC ATT GAC TAT CAT T-3′, and amplified a 1,572-bp fragment. In parallel, we analyzed chicken β-actin as an internal control for normalization. The forward and reverse primers, 5′-CCC TGA ACC CCA AAG CCA AC-3′ and 5′-GAC TCC ATA CCC AAG AAA GA-3′, respectively, produced a predicted 487-bp fragment between positions 394 and 880. The conditions for cDNA amplification have been described previously (30).

RNA Isolation and Taqman Analysis

RNA from LMH cells was isolated with the RNeasy Kit (QIAGEN AG, Basel, Switzerland). One microgram of total RNA was reverse-transcribed with the Moloney murine leukemia virus reverse transcriptase (Roche Molecular Biochemicals). PCR was performed using the Taqman PCR Core Reagent Kit (PE Applied Biosystems, Rotkreuz, Switzerland) and the transcript level quantitated with an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Rotkreuz, Switzerland) according to the manufacturers 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 PCR reactions: 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′; CYP3A37: probe, 5′-TTG GCC CAG GAA TGC CCA GCT-3′; forward primer, 5′-GTC CCA AAG AAA GGC AAT GGT 3′; reverse primer, 5′-GGC CAT TTG GGT TGT TCA AG-3′; ALAS: probe, 5′-TTC CGC CAT AAC GAC GTC AAC CAT CTT- 3′; forward primer, 5′-GCA GGG TGC CAA AAC ACA T-3′; reverse primer, 5′-TCG ATG GAT CAG ACT TCT TCA ACA-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, CYP3A37, ALAS, and GAPDH transcript levels were measured in separate tubes, and GAPDH was used for normalization of the CYP2H1, the CYP3A37, and the ALAS values.

Western Blot Analysis

Chicken embryo livers were frozen in liquid nitrogen and crushed in a mortar. The tissue was resuspended in 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride (lysis buffer) and sonicated three times for 10 sec on ice. The extract was centrifuged at 4 C for 20 min at 14,000 rpm. Chick embryo hepatocytes were cultured and prepared as previously described (53, 54). Primary hepatocytes and LMH cells were washed twice with PBS, resuspended in lysis buffer, sonicated, and centrifuged as described above. The protein concentration of the supernatant was determined using the ESL protein assay (Roche Molecular Biochemicals). Equal amounts of protein were separated on 10% Tricine-SDS-PAGE. The proteins were blotted onto a NYTRAN 13 N membrane (Schleicher & Schuell, Inc., Dassel, Germany) using a Multiphor II Nova Blot (Pharmacia LKB, Dübendorf, Switzerland) and 39 mM glycine, 48 mM Tris, 0.0375% SDS, and 20% methanol for semidry transfer of the proteins at 0.8 mA/cm^2 for 1 h. Proteins were visualized according to the enhanced chemiluminescence protocol (Amersham Pharmacia Biotech, Zürich, Switzerland) using an anti-CYP2H1 antibody. This polyclonal rabbit antibody was generously provided by Dr. P. Sinclair (VA Hospital, White River Junction, VT).

Northern Blot Analysis

Total RNA (10 μg) was subjected to electrophoresis on a 1% agarose/formamide gel. RNA was transferred to a NYTRAN 13 N membrane (Schleicher & Schuell, Inc.) overnight in 10× SSC (1.5 M NaCl, 150 mM sodium citrate) and was cross-linked to the membrane by UV exposure for 12 sec. Prehybridization was carried out in 50% formamide, 5× SSC, 5× Denhardt's solution, 1% SDS, and 10% (wt/vol) dextran sulfate. Hybridization probes were generated by PCR amplification using the primers mentioned above and labeled with [α-^{32}P]dATP using the Random Primed DNA Labeling Kit (Roche Molecular Biochemicals). The probes were boiled for 5 min in 500 μl of 10 mg/ml salmon sperm DNA and chilled on ice. Hybridization was carried out for 16–20 h at 42 C. Washes were performed in 2× SSC/0.1% SDS at room temperature for 30 min and in 2× SSC/0.1% SDS at 65 C for 20 min. Membranes were exposed to x-ray film using intensifying screens or to PhosphorImager screens for 12–48 h.

EMSAs

Chicken CXR and chicken RXRγ were synthesized *in vitro* using the TNT T7 Quick Coupled Transcription/Translation System (Promega Corp., Catalys AG, Wallisellen, Switzerland) according to the manufacturer's instructions. Chicken RXRγ was chosen for these experiments because RXRγ is the only chicken RXR ortholog known so far. Probes were labeled with Klenow enzyme in the presence of radiolabeled ^{32}P-α-ATP, and the probe was purified over a Biospin 30 Chromatography Column (Bio-Rad Laboratories, Inc., Glattbrugg, Switzerland). A volume of labeled oligonucleotide corresponding to 100,000 cpm was used for each reaction in 10 mM Tris (pH 8.0), 40 mM KCl, 0.05% NP-40, 6% glycerol, 1 mM dithiothreitol, 0.4 μg/μl BSA, 0.2 μg poly(dI-dC)*poly(dI-dC), and 2.5 μl of each of the *in vitro* synthesized proteins as described previously (22). To test for supershifts, 0.5 μl of monoclonal antimouse-RXR rabbit antibody (kindly provided by Dr. P. Chambon, IGBMC, Université Louis Pasteur, Illkirch, France) was added to the reaction mix. This antibody has been positively tested for cross-reaction with the chicken RXRγ in Western blots (data not shown). The mix was incubated for 20 min at room temperature and subsequently electrophoresed on a 6% polyacrylamide gel in 0.25× Tris-Borate-EDTA buffer followed by autoradiography at −70 C.

Transcriptional Activation Assays

To perform transactivation assays, CV-1 cells were kept in DMEM/F12 medium without phenol red, supplemented with

10% charcoal-stripped FBS, and plated in six-well dishes at a density of 625,000 cells per well. A total of 2.5 μg DNA per well, including 150 ng of receptor expression vector, 400 ng of CAT reporter gene plasmid, 800 ng pSV-β-galactosidase expression vector (Promega Corp.) and carrier plasmid were transfected and cells were exposed to drugs. Cell extracts were prepared and assayed for CAT using a CAT-ELISA kit (Roche Molecular Biochemicals). β-Galactosidase activities were determined. CAT concentrations were then normalized against β-galactosidase values to compensate for varying transfection efficiencies as described previously (22).

Acknowledgments

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