CXR, a novel xenobiotic-sensing orphan nuclear receptor is related to both mammalian pregnane X receptor (PXR) and constitutive androstane receptor (CAR)

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Abbreviations: CYP, cytochrome P-450; CXR, chicken xenobiotic receptor; PXR, pregnane X receptor; CAR, constitutive androstane receptor; PBRU, phenobarbital-responsive unit; PB, phenobarbital; RXR, 9-cis retinoic acid receptor; DR, direct repeat; PIA, propylisopropylacetamide; PCN, 5-pregnen-3β-ol-20-one-16α-carbonitrile; CAT, chloramphenicol acetyltransferase

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Abstract

Nuclear receptors comprise a large family of ligand-modulated transcription factors that mediate cellular responses to small lipophilic molecules including steroids, retinoids, fatty acids and exogenous ligands. Orphan nuclear receptors with no known endogenous ligands have been discovered to regulate of drug-mediated induction of cytochromes P-450 (CYP), the major drug-metabolizing enzymes. Here, we report the cloning of a new orphan nuclear receptor from chicken, termed chicken xenobiotic receptor (CXR), that is closely related to two mammalian xenobiotic-activated receptors, the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR). Expression of CXR is restricted to tissues where drug-induction of CYPs predominantly occurs, namely liver, kidney, small intestine and colon. Furthermore, CXR binds to a previously identified phenobarbital-responsive enhancer unit (PBRU) in the 5'-flanking region of the chicken CYP2H1 gene. A variety of drugs, steroids and chemicals activate CXR in CV-1 cell transactivation assays. The same agents induce PBRU-dependent reporter gene expression and CYP2H1 transcription in a chicken hepatoma cell line. These results provide convincing evidence for a major role of CXR in the regulation of CYP2H1 and add a new member to the family of xenobiotic-activated orphan nuclear receptors.

Introduction

Members of the CYP gene superfamily encode for heme-proteins involved the in oxidative metabolism of xenobiotic and endogenous substrates in all species (1). In vertebrates, this biotransformation of drugs, steroids and other compounds occurs predominantly in the liver whereas extrahepatic tissues such as intestine, kidney, skin, lung or brain play a less prominent role (2, 3). Expression of some of these CYPs can be transcriptionally regulated by their substrates or other compounds (2-5). Phenobarbital (PB) and other drugs of this class of prototypical inducers displays a distinct activation pattern of CYPs. PB-type inducers are a family of compounds that are structurally diverse but share the common general characteristics of being small and lipophilic molecules (3).

Steroid hormones possess similar properties in regard to size and hydrophobicity which enables them to diffuse into cells and interact with specific intracellular receptors (6, 7). Steroid hormone receptors belong to the superfamily of nuclear receptors with conserved structural features. Also included in this superfamily are receptors with non-steroid ligands such as thyroid hormone, retinoids or fatty acids (8-10). Nuclear receptors with known endogenous ligands are distinguished from so-called orphan nuclear receptors for which true endogenous ligands have not been identified (6, 8, 11). Steroid hormone receptors bind to cognate DNA recognition elements as homodimers in contrast to most of the non-steroid nuclear receptors which heterodimerize with the 9-cis retinoic acid receptor (RXR, NR2B group) in order to bind to DNA (7, 11). The recognition elements of steroid hormone receptors consist usually of two hexamer half-sites arranged as a palindrome. Non-steroid nuclear receptors preferentially bind to two hexamer half-sites organized as direct repeat (DR) spaced by a variable number of nucleotides (8, 11).

A link between CYP regulation and nuclear receptors has been established with the discovery of the role of several orphan nuclear receptors in the induction of CYPs by xenobiotics. In

particular, PXR (NR1I2) and CAR (NR1I3) have been shown to play crucial roles in induction of members of the CYP3A and CYP2B subfamilies in human and mouse liver, respectively (reviewed in refs. 12-15). PXR and CAR are activated by the same compounds that induce the respective CYPs and bind as heterodimers with RXR to response elements in 5'-enhancer regions of these genes. Xenobiotic-responsive elements have previously been defined in rat CYP2B2 and CYP3A1, mouse Cyp2b10 and human CYP2B6 and CYP3A4 (reviewed in refs. 13, 15 and 16). These elements are characterized by putative nuclear receptor recognition sites.

Chicken hepatocytes and, more recently, chicken hepatoma cells have been used extensively as a model for elucidating the molecular mechanism of xenobiotic induction (for recent examples, see refs. 17 and 18). In chicken liver, CYP2H1 has been identified as one of the major PB-inducible genes and a PBRU in its 5'-flanking region has been characterized (17, 19). We have shown that a DR-4 element in combination with a nuclear factor-1 (NF-1) site within the CYP2H1 264-bp PBRU is required for the response to drugs (19). Similar arrangements of response elements are conserved in mouse, rat and human PBRUs. Moreover, drug-activation of human and mouse PBRUs in a chicken hepatoma cell line (LMH) strongly suggests conservation of the molecular induction mechanisms between chicken and mammals (19).

In this report, we describe the cloning and functional analysis of a novel chicken orphan nuclear receptor that is closely related to the NR1I subfamily of mammalian xenobiotic receptors. We have designated the new receptor "chicken xenobiotic receptor" (CXR, NRXXX) because it responds to numerous drugs and chemicals and activates a xenobiotic-metabolizing enzyme, chicken CYP2H1. We demonstrate that this receptor mediates the transcriptional control of CYP2H1 by various drugs and chemicals via the recently described enhancer unit. Sequence comparison and activation profiles suggest a close relationship of this transcription factor to the mammalian drug-sensing orphan nuclear receptors PXR and CAR.

Materials and Methods

Reagents. Dexamethasone, metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone), 5-pregnen-3β-ol-20-one-16α-carbonitrile (PCN), rifampicin and clotrimazole (1-[o-chlorotrityl]-imidazole) were obtained from Sigma, Buchs, Switzerland. Propylisopropylacetamide (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 (mifepristone) 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. Phenobarbital sodium salt (5-ethyl-5-phenyl-barbituric acid sodium salt) was purchased from Fluka, Buchs, Switzerland. Poly(dI-dC)*poly(dI-dC) was from Amersham Pharmacia Biotech, Dübendorf, Switzerland. All other reagents and supplies were obtained from standard sources. Cell culture media, sera and tissue culture reagents were purchased from Life Technologies, Basel, Switzerland unless noted otherwise.

Plasmids. Generation of the reporter plasmid pBLCAT5 containing either the CYP2H1 264-bp PBRU or the CYP2H1 264-bp double mutant has previously been described (19). CXR and chicken RXRγ coding regions (20) were generated by PCR, sequenced and subsequently cloned into the expression vector pSG5 (Stratagene Europe, Amsterdam, The Netherlands).

Culture and transfection of LMH cells. LMH cells were obtained from the American Type Culture Collection 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 (19).

Isolation of total and poly-A RNA. Tissues from an adult female chicken were frozen in liquid nitrogen and homogenized with a glass-Teflon homogenizer at 4°C in TRIZOL reagent (Life Technologies, Basel, Switzerland) and total RNA from both chicken tissues and LMH cells was obtained according to the suppliers manual. Poly-A RNA was subsequently prepared from total RNA with the Oligotex mRNA Midi Kit (Qiagen, Basel, Switzerland) following the protocol provided by the manufacturer.

Northern analysis. The 820-bp fragment that was used in screening the chicken liver cDNA library described below was ^{32}P-labeled with the Random Primed DNA Labeling Kit (Roche Molecular Biochemicals, Rotkreuz, Switzerland) in a Klenow reaction using random hexamer primers followed by purification over a Biospin 6 Chromatography Column (Biorad, Glattbrugg, Switzerland). 20μg of total RNA or 15μg of poly-A RNA were loaded onto a formaldehyde/agarose gel. Northern blotting was carried out according to standard techniques (21).

Gel-mobility shift assays. Chicken CXR and chicken RXRγ were synthesized in vitro using the TNT T7 Quick Coupled Transcription/Translation System (Promega, Catalys AG, Wallisellen, Switzerland) according to the manufacturer's instructions. Probes were labeled with Klenow enzyme in the presence of radiolabeled ^{32}P-α-ATP and the probe was purified over a Biospin 6 Chromatography Column (Biorad, Glattbrugg, Switzerland). A volume of labeled oligonucleotide corresponding to 100'000 cpm was used for each reaction in 10mM Tris (pH 8.0), 40mM KCl, 0.05% NP-40, 6% glycerol, 1mM dithiothreitol, 0.2μg poly(dI-dC)*poly(dI-dC) and 2.5μl of the *in vitro* synthesized proteins as described previously (22). To test for supershifts, 0.5μl of monoclonal anti-mouse-RXR rabbit antibody (kindly provided by Dr. P. Chambon, IGBMC, Université Louis Pasteur, Illkirch, France) were added to the reaction mix. This antibody has been positively tested for cross-reaction with the chicken RXRγ in Western

blots (data not shown). Cold oligonucleotides for competition experiments were added at a 20-fold molar excess. The mix was incubated for 20 minutes at room temperature and subsequently electrophoresed on a 6% polyacrylamide gel in 0.5x Tris-Borate-EDTA buffer followed by autoradiography at –70°C.

Transcriptional activation and CAT reporter gene assays. To perform transactivation assays, CV-1 cells were kept in DMEM/F12 medium without phenol red, supplemented with 10% charcoal-stripped fetal bovine serum and were plated in 6-well dishes at a density of 625'000 cells per well. A total of 2.5μg DNA per well, including 150ng of receptor expression vector, 400ng of chloramphenicol acetyltransferase (CAT) reporter gene plasmid, 800ng pSV-β–galactosidase expression vector (Promega, Catalys AG, Wallisellen, Switzerland) and carrier plasmid were transfected and cells exposed to drugs as described in ref. 22. Cell extracts were prepared and assayed for CAT using a CAT-ELISA kit (Roche Molecular Biochemicals, Rotkreuz, Switzerland). β-Galactosidase activities were determined according to ref. 23. CAT concentrations were then normalized against β-galactosidase values in order to compensate for varying transfection efficiencies.

Semiquantitative PCR with the Taqman system. One μg of total RNA was reverse-transcribed with the MMLV reverse transcriptase (Roche Molecular Biochemicals, Rotkreuz, Switzerland). 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). CYP2H1 and glyceraldehyde 3-phosphate dehydrogenase transcript levels were measured in separate tubes and glyceraldehyde 3-phosphate dehydrogenase was used for normalization of the CYP2H1 values as described previously (19).

Results

Isolation and classification of the chicken orphan nuclear receptor CXR. The cDNA sequences of human and mouse PXR and CAR, respectively, were aligned using the multiple alignment algorithm BLOCKS (24) in order to identify conserved sequence stretches. With the CODEHOP program, we subsequently screened the aligned sequence stretches for degenerate, 5'-clamped primers accounting for chicken codon usage (25). The primers 5'-GCC TGC CGG CTG CAR AAR TGY YT-3' and 5'-GGC CCA TCA GCT TGG CRT ANA RRA A-3' amplified an 820-bp fragment in a hot-start, touchdown PCR with LMH cDNA as template. This fragment was subcloned and used as a probe in the screening of a chicken liver Lambda ZAP phage cDNA library (Stratagene Europe, Amsterdam, The Netherlands). Two different clones were obtained, subcloned and sequenced. One clone contained a partial sequence with high homology to mammalian orphan nuclear receptors whereas the other clone consisted of a different, full-length orphan nuclear receptor cDNA. This full-length clone was denominated "CXR" and its DNA and deduced amino acid sequences are depicted in Fig. 1A where the DNA-binding domain (DBD) is boxed and the conserved cysteins responsible for forming zinc-finger domains (26) are shaded. The DBD and ligand-binding domain (LBD) of CXR were compared to those of other receptors using the GAP algorithm included in the Wisconsin Package Version 10.0 software (Genetics Computer Group (GCG), Madison, WI). Similarity between DBDs of CXR and other nuclear receptors including mammalian PXRs (27) and CARs (28, 29), xenopus benzoate X receptor (BXR, NR1I2, ref. 30) and chicken 1,25-dihydroxyvitamin D_3 receptor (NR1I1, ref. 31) was between 61% and 67% amino acid identity (Fig. 1B). By comparison, the DBDs of chicken progesterone receptor (PR, NR3C3) and estrogen receptor (ER, NR3A1) revealed 44.6% and 59.1% amino acid identity with CXR, respectively. The highest similarity to the LBD of CXR was observed in human CAR with 56.3% amino acid identity. All of the PXRs and mouse CAR exhibit a similarity of about 50% to CXR. When comparing the CXR LBD to

the xenopus benzoate X receptor and the chicken 1,25-dihydroxyvitamin D_3 receptor, similarities dropped to 44.1% and 40.1%, respectively. Moreover, the chicken progesterone and estrogen receptor LBDs displayed weak similarity of about 23%. These comparisons position the chicken CXR into the NR1 subfamily of orphan nuclear receptors (8, 10). The same conclusion was reached when generating phylogenetic trees from multiple alignments of full length amino acid sequences with the ClustalW program (Fig. 1C and D). Apart from the receptors used in Fig. 1B, the chicken RZRβ (NR1F2), thyroid hormone receptor (NR1A group), RARγ (NR1B3), RARα (NR1B1), SF-1 (NR5A1), COUP (NR2F group) and RXRγ (NR2B3) were included in the trees. The branch lengths of the phylogram (Fig. 1C) and the unrooted tree (Fig. 1D) were calculated from the number of amino acid substitutions per site. The phylogram depicting the chicken CXR within a selection of nuclear receptors also positions CXR into the nuclear receptor subfamily NR1 (8, 10). Together, these results suggest that CXR is closely related to mammalian drug-responsive orphan nuclear receptors of the NR1I group (10).

Chicken CXR is expressed in the main drug-metabolizing tissues. CXR tissue expression was examined by Northern blot analysis of various adult chicken tissues (Fig. 2A). Expression of CXR was detected in liver, kidney, small intestine and colon but not in any of the other tissues (Fig. 2A). Expression levels in liver and kidney were high whereas in small intestine and colon, only a faint signal could be detected. Two bands with sizes of about 2kb and 1.4kb, respectively, could be observed in both liver and kidney, whereas only the 1.4kb band was observed in small intestine and colon. In liver, the 2kb transcript was more abundant than the 1.4kb mRNA while in kidney, the 1.4kb band was more prominent than the 2kb mRNA. The chicken LMH hepatoma cell line exhibited similar expression levels when compared to adult chicken kidney (Fig. 2B). The 1.4kb transcript was clearly detectable in total RNA of LMH cells whereas only a very weak signal could be detected for the 2kb band. No change in the level of CXR mRNA was

observed in LMH cells after 24 hours of drug-induction with either vehicle, 400μM PB, 50μM dexamethasone or 400μM metyrapone (Fig. 2B).

CXR binds to the wildtype chicken CYP2H1 264-bp PBRU. A PB-responsive unit has been identified in the chicken CYP2H1 5'-flanking region and a conserved nuclear-receptor recognition element has been shown to confer PB-inducibility in this element (19). This nuclear receptor recognition element consists of two hexameric core motifs separated by four nucleotides, a so-called direct repeat (DR-4). Mutations in either hexamer half-site or in both half-sites of this DR-4 abolish PB-induction by this element in reporter gene assays (19). The sequences of both the wildtype DR-4 and the double mutation within the 264-bp PBRU are shown in Fig. 3A. As nuclear receptors of the NR1 subfamily have been shown to interact predominantly with direct repeat elements (8) and CXR was found to be a member of this class, a gel-mobility-shift assay was used to determine if CXR binds to the PB-responsive element from chicken CYP2H1. Neither *in vitro* transcribed/translated chicken CXR nor chicken RXRγ alone bound to the radiolabeled 264-bp PBRU (Fig. 3B, lanes 2 and 3). Only the CXR-RXRγ heterodimer binds to the PBRU and this complex could be supershifted with antibodies against RXR (Fig. 3B, lanes 4 and 5). A 20-fold molar excess of cold, unlabeled 264-bp PBRU competed with the radiolabeled probe for CXR/RXRγ binding and therefore reduced the shift. Unlabeled double mutant 264-bp PBRU competed only weakly with the wildtype 264-bp PBRU for CXR/RXRγ binding (Fig. 3B, lanes 6 and 7) further substantiating the importance of the DR-4 element in the 264-bp PBRU.

Chicken CXR activates the CYP2H1 264-bp PBRU in CV-1 cells. Transactivation assays using a selection of prototypical inducers drugs in CV-1 cells were performed to test whether CXR activates the 264-bp PBRU in correlation with the results from gel-mobility shift assays. Previous studies have employed a wide variety of drugs to investigate nuclear receptor and CYP

activation in chicken and mouse (19, 32). Accordingly, we chose PB (400μM) and the PB-like inducers PIA (250μM), glutethimide (500μM) and TCPOBOP (10μM), a potent inducer in mouse. For comparison, dexamethasone (50μM), metyrapone (400μM) and RU486 (10μM), three common CYP3A inducers, and the prototypical CYP1A1 inducer β-naphtoflavone (10μM) were also tested. Furthermore, we were interested in the effects of PCN (50μM) and rifampicin (100μM) because of their species-specific effects on PXR activation and CYP3A expression. Clotrimazole (10μM) was chosen because it has profound effects on mouse and human PXR and CAR (32). CV-1 cells were transfected with chicken CXR and the 264-bp PBRU in a CAT reporter gene vector and treated with drugs for 24 hours. None of the drugs had an effect on reporter gene expression in absence of CXR (data not shown). The classical PB-type inducers all activated CXR and thereby the reporter gene constructs between 2.3- and 3.3-fold (Fig. 4A). Metyrapone was the most potent inducer from the class of typical CYP3A inducers showing 3.8-fold induction. RU486, dexamethasone, PCN and rifampicin however were limited to less than 1.8-fold activation. The known CYP1A1 inducer β-naphtoflavone also strongly activated CXR 2.9-fold which exceeds the effect found after PB treatment. Finally, clotrimazole was the most potent inducer of CXR in these assays resulting in 6-fold induction (Fig. 4A).

Next, we wanted to investigate the role of CXR on the response element by comparing its activation pattern in CV-1 cells to activation of PBRU reporter gene and transcriptional activation of CYP2H1 mRNA in a physiological environment. LMH cells were therefore transiently transfected with a 264-bp PBRU reporter gene plasmid and were treated with the same drugs as mentioned above for 16 hours. CAT levels were measured and normalized against total protein content of the differently treated LMH cells. Relative induction levels of CAT revealed high activation of the 264-bp PBRU with PB, metyrapone, PIA and glutethimide. Dexamethasone, β-naphtoflavone, PCN and rifampicin had only minor or no effects. These results have in part been published previously (19) and are listed here for comparison. In addition, clotrimazole was one of the most potent inducers of the 264-bp PBRU in LMH cells

mediating a 20-fold induction. These findings are similar to those for metyrapone, PIA and glutethimide. Moreover, RU486 was a weaker inducer (7-fold) when compared to clotrimazole whereas TCPOBOP elevated reporter gene levels only 2.7-fold (Fig. 4B).

With the 264-bp PBRU being only a small part of the CYP2H1 5'-flanking region, overall response of these inducers on CYP2H1 mRNA levels was of interest. All of the compounds induced CYP2H1 mRNA after 16 hours of drug-exposure. Metyrapone, PIA and glutethimide were the strongest activators mediating a 18- to 25-fold increase in CYP2H1 mRNA (Fig. 4C). PB and RU486 moderately elevated CYP2H1 transcript levels 12-fold and the remainder of the drugs displayed resulted in only about 6-fold activation (Fig. 4C). Taken together, the transactivation assays provide unequivocal evidence that CXR is a xenobiotic-responsive receptor and that CXR is the major mediator of xenobiotic response through the CYP2H1 PBRU.

Discussion

In this report, we describe the cloning and functional analysis of CXR, a novel chicken xenobiotic-sensing receptor. CXR shows all of the structural features typical for a member of the nuclear receptor superfamily including a conserved DNA-binding domain with two zinc-finger domains (26) and a conserved ligand-binding domain (8). CXR heterodimerizes with chicken RXRγ in order to bind to DNA, as evidenced in gel-mobility shift assays. This binding occurs on a repeat of hexamer-half-sites derived from the AG(G/T)TCA consensus sequence characteristic for all nuclear receptors (8) and binding is abolished by site-directed mutagenesis of these half-sites. Moreover, these hexamer-half-sites are organized as a direct repeat with a four-basepair spacer (DR-4), preferentially bound by non-steroid liganded nuclear receptors (11). The apparent lack of an endogenous ligand specifies the CXR at this time to be an orphan nuclear receptor (8, 11).

In adult chicken, CXR is expressed in liver, kidney, small intestine and colon. The same tissues are known for their role in drug-metabolism and xenobiotic induction of multiple CYP genes which constitute the major drug-metabolizing enzyme system (2, 3). In the Northern blot, two mRNA bands were detected that showed different distribution in liver compared to kidney and intestine. In liver, a 2kb transcript was more abundant when compared to a 1.4kb mRNA whereas the ratio was reversed in kidney. Whether these two bands are splice-variants of CXRs or represent different, but closely related receptors remains to be investigated. Interestingly, a distribution pattern similar to that observed in kidney and intestine was observed in the chicken hepatoma cell line LMH. This might be due to the transformed properties of the cell line or to its clonal selection. In contrast, chicken liver obviously represents a mixture of different cell types of which only a fraction are drug-responsive.

The binding of CXR as heterodimer with chicken RXRγ to a recently identified PBRU from chicken CYP2H1 (19) suggest an essential role of CXR in CYP regulation. Therefore, in search

of activators of CXR, we tested a variety of compounds known to induce different CYPs. The activation pattern of CXR in CV-1 cells transactivation assays closely correlated with the activation of the 264-bp PBRU in reporter gene assays in LMH cells and paralleled drug-effects on CYP2H1 mRNA. Thus, CXR clearly represents the major receptor involved in activating the CYP2H1 264-bp PBRU. CYP2H1 mRNA was also induced by some compounds that were inactive in reporter gene assays suggesting additional transcriptional control of CYP2H1 by other orphan nuclear receptors and other recognition sites in the CYP2H1 5'-flanking region.

Sequence comparison of the DBD and LBD of CXR to other nuclear receptors surprisingly revealed almost equal amino acid identity to several receptors of the group NR1I. This group presently consists of the 1,25-dihydroxyvitamin D_3 receptor, the xenopus benzoate X receptor and of the mammalian xenobiotic-sensing receptors PXR and CAR. Among these, CXR displays higher similarity to PXRs and CARs when comparing the full-length amino acid sequences. Calculation of the average evolutionary distance between CXR and human and mouse CARs revealed 0.474 amino acid substitutions per site and was slightly smaller than the distance between CXR and mammalian PXRs (0.52 amino acid substitutions per site). By comparison, the average distance between PXRs and CARs is 0.55 amino acid substitutions per site. Therefore, CXR is almost equally related to PXRs and CARs at the amino acid sequence level. This raises the question of whether CXR represents an ortholog of mammalian PXRs or CARs or, alternatively, a new class of xenobiotic-sensing receptors. Usually, nuclear receptor orthologs share more than 90% amino acid identity in their DBD and LBD (8). This is different for the xenobiotic-sensing receptors PXRs and CARs where the LBD share only about 70% identity among mammals alone (32). The CXR sequence revealed 61% to 67% amino acid identity in the DBD and 40% to 56% identity in the LBD when compared to other members of the NR1I group of nuclear receptors.

However, the evolutionary distance between CXR, PXR and CAR deviates considerably from that of other chicken orthologs of nuclear receptors. Thus, the similarities between the chicken

and mammalian farnesoid X receptor (FXR) and liver X receptor (LXR), the receptors for bile-acids and oxysterols, respectively, are around 94% and 86% amino acid identity in the DBD and about 86% and 83% identity in the LBD, respectively (R. Amherd and U. A. Meyer, unpublished results).

We conclude that CXR may well be a new type of xenobiotic-sensing receptors with potential orthologs in mammalian genomes. Alternatively, CXR might represent the ancestral gene that diverged into CAR and PXR in the course of evolution as an adaptive response to different environmental and nutritional pressures. In any case, the basic mechanism of drug-induction of enzymes involved in drug-metabolism by orphan nuclear receptors apparently is evolutionary conserved over the 300 million years that separate birds from humans.

Acknowledgments

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Figure Legends

FIG. 1. CXR is closely related to mammalian drug-responsive orphan nuclear receptors. (A) Nucleotide and predicted amino acid sequence of CXR. The putative DBD is boxed and the conserved cysteins responsible for forming zinc-finger domains are shaded. (B) Amino acid comparison between CXR and a selection of nuclear receptors. The similarity of the DBDs and LBDs between CXR and the selected nuclear receptors is indicated as percentage amino acid identity. (C) Phylogram of full length amino acid sequences of CXR and related nuclear receptors. (D) Unrooted phylogenetic tree of full length amino acid sequences of CXR and mammalian CARs and PXRs, respectively.

The scale-bars for distance measurements represent 0.1 amino acid substitutions per site.

FIG. 2. Expression pattern of CXR. (A) Northern blot analysis of adult chicken tissue. 15μg of poly-A RNA were used for all tissues. (B) Northern blot analysis of LMH cells. LMH cells were induced with either vehicle (Contr), phenobarbital (PB), dexamethasone (Dex) or metyrapone (Met) and total RNA was isolated of which 20μg were used for Northern blots.

RNA size markers in number of bases are shown to the left of the blots.

FIG. 3. CXR binds to the CYP2H1 264-bp PBRU. (A) Sequences of the wildtype DR-4 element within the 264-bp PBRU and the double mutant with knocked-out hexamer half-sites as adapted from ref. 19. (B) Gel-mobility shift assay. Radiolabeled 264-bp PBRU was incubated with *in vitro* transcribed/translated CXR (lane 3-7), chicken RXRγ (lane 2 and 4-7), anti-RXR antibody (lane 5), cold 264-bp PBRU in 20-fold molar excess (lane 6) and cold 264-bp PBRU double mutant in 20-fold molar excess as competitors (lane 7), as indicated. Arrows depict the unbound probe (c), the complex of CXR, RXRγ and the probe leading to a shift (b) and the complex of CXR, RXRγ, anti-RXR antibody and the probe resulting in a supershift (a).

FIG. 4. Activation of CXR by different drugs. (A) CV-1 cells were co-transfected with an expression plasmid for CXR together with a CAT reporter gene plasmid containing the CYP2H1 264-bp PBRU. Cells were then treated either with vehicle or drugs for 24 hours. Cell extracts were analyzed for CAT expression normalized against β-galactosidase levels. (B) Activation pattern of the CYP2H1 264-bp PBRU. LMH cells were transfected with the 264-bp PBRU in a CAT reporter gene plasmid and induced for 16 hours with the same compounds in Fig. 4A. CAT levels were determined and normalized against non-treated control cells. Except for the previously unpublished results of clotrimazole, RU486 and TCPOBOP, data are adapted from ref. 19. (C) Activation pattern of CYP2H1 mRNA. LMH cells were treated with the compounds used in Fig. 4A and B for 16 hours. Total RNA was isolated and reverse-transcribed. Relative mRNA levels of CYP2H1 standardized against glyceraldehyde 3-phosphate dehydrogenase levels were obtained by semiquantitative PCR using the Taqman System.

Values are the average of three independent experiments and error bars represent standard deviations.

FIG. 1

A

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1    CGTGGGGACACAACCGCAGCGGTGACACCGGGCACAGCATCCCAGCCATGTCCCAGTCCA  60
61   GCCCCTCGGACCCGGACAGCCCCGGGGCGCAGCGTTGTCCCAACGTCACCGATGTCACCG  120
                                                  M  S  Q  S  S
121  AGGAGGAGCTGAAGGTGTGCGCTGTGTGCGGGGACAGAGCCACCGGGTACCACTTCCACG  180
      P  S  D  P  D  S  P  G  A  Q  R  C  P  N  V  T  D  V  T  E
181  TGATGAGCTGCGAAGGGTGTAAAGGGTTCTTCAGGCGCTCCATCCTTAAGGGTGTGCACT  240
      E  E  L  K  V  C  A  V  C  G  D  R  A  T  G  Y  H  F  H  V
241  TCACCTGCCCCTTCACCCGGAGCTGCCCCATAAACCAAAGCCAAGCGCCGGCAGTGCCAGG  300
      M  S  C  E  G  C  K  G  F  F  R  R  S  I  L  K  G  V  H  F
301  CGTGCCGCCTCCAGAAGTGCCTCGACGTGGGGATGCGGAAGGACATGATCATGTCGGAGG  360
      T  C  P  F  T  R  S  C  P  I  T  K  A  K  R  R  Q  C  Q  A
361  AGGCCCTGGGCCGCCGGCGGGCGCTGAGGCTGCAGAGGAGGTTGGCGCAGGCACAGCCCG  420
      C  R  L  Q  K  C  L  D  V  G  M  R  K  D  M  I  M  S  E  E
421  GGGGGCTGACAGCGGAAGGTGGCCCTCATCAGCATCCTCATCGCCGCGCACAAACGCA  480
      A  L  G  R  R  R  A  L  R  L  Q  R  R  L  A  Q  A  Q  P  G
481  CCTTCGACTCCAGCTTCTCCAGTTCCAGCACTACCAGGCTTCCGTGCGCCTCTGCATCC  540
      G  L  T  A  E  Q  Q  E  L  I  S  I  L  I  A  A  H  K  R  T
541  CCGGCCCGTGCTCCCAGAGCCCACCCGGACCGGGCGTCCCTTCGGCATCCTTGCCCCAG  600
      F  D  S  S  F  S  Q  F  Q  H  Y  Q  P  A  V  R  L  C  I  P
601  AGTTGGACTGTGTCTGGATGAGGATGTGCTGCCCGAGCGTCTTCTTCCATCCTGCCCAC  660
      G  P  C  S  Q  S  P  P  G  P  G  V  P  S  A  S  L  S  P  Q
661  CCGACCTCAGCACCTTCATGATCCAGCAGGTCATCAAATTCGCCAAGGAGATCCCGGCGT  720
      L  D  C  L  D  E  D  V  L  P  D  V  F  S  I  L  P  H  F  A
721  TCAGGGGGCTGCCCATCGACGACCAGATCTCTCTGCTCAAGGGTGCCACGCTGGGGATCT  780
      D  L  S  T  F  M  I  Q  Q  V  I  K  F  A  K  E  I  P  A  F
781  GCCAGATCCAGTTCAACACCGTGTTCAACGAGGAGACCAACGCTGGGAGTGCGGGCAGC  840
      R  G  L  P  I  D  D  Q  I  S  L  L  K  G  A  T  L  G  I  C
841  ACTGCTTCACCATCAAGGATGGGGCTCTGGCCGGCTTCCAGCAGATCTACCTGGAGCCAC  900
      Q  I  Q  F  N  T  V  F  N  E  E  T  N  A  W  E  C  G  Q  H
901  TGCTCAAATTCCACATCAGCCTGAAGAAGCTGCGGCTGCACGAGGCCGAGTACGTCCTGC  960
      C  F  T  I  K  D  G  A  L  A  G  F  Q  Q  I  Y  L  E  P  L
961  TGGTGGCCATGCTGCTCTTCTCGCCAGACCACGCCAGCGTCACCCAGCGGGATTTCATTG  1020
      V  A  M  L  L  F  S  P  D  H  A  S  V  T  Q  R  D  F  I  D
1021 ACCAGCTGCAGGAGAAGGTGGCCCTCACCCTCAAGAGCTACATCGACCACCGGCACCCCA  1080
      Q  L  Q  E  K  V  A  L  T  L  K  S  Y  I  D  H  R  H  P  M
1081 TGCCCGAGGGCAGGTTCCTCTACGCCAAACTGCTGCTGCTCTGACGGAGCTGCAGACAC  1140
      P  E  G  R  F  L  Y  A  K  L  L  L  L  L  T  E  L  Q  T  L
1141 TGAAGATGGAGAACACGGCAGATCTGCTCCACACATCCAGGACCTCTCCAGCATGACTCCGC  1200
      K  M  E  N  T  R  Q  I  L  H  I  Q  D  L  S  S  M  T  P  L
1201 TGCTCTCCGAAATCATCAGCTGAAGGCCACCAAGGCACTGAGCCCCCATCCCGCGCACG  1260
      L  S  E  I  I  S  *
1261 ACGCGGGGCTCCATCGCACTTTACAGGGCAGAGTAAAGCTCTTTTATTTTTAAAAAAAAA  1320
1321 AAAAAAAAAAAAAAAAAAAA                                          1340
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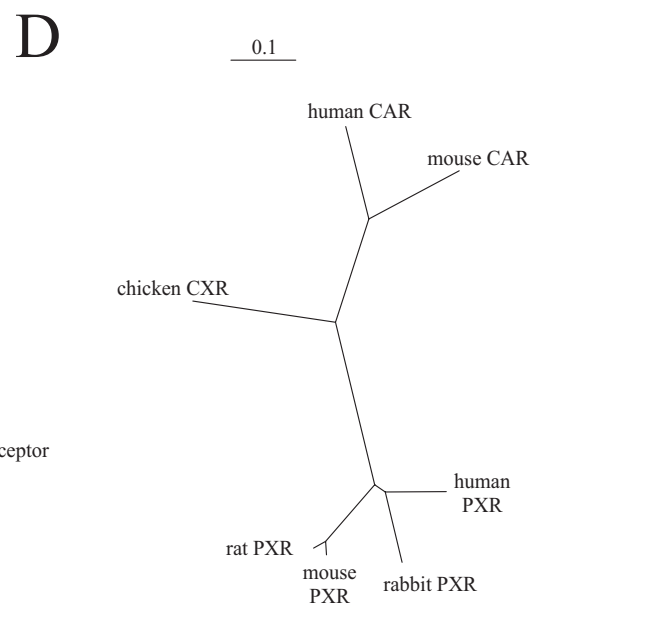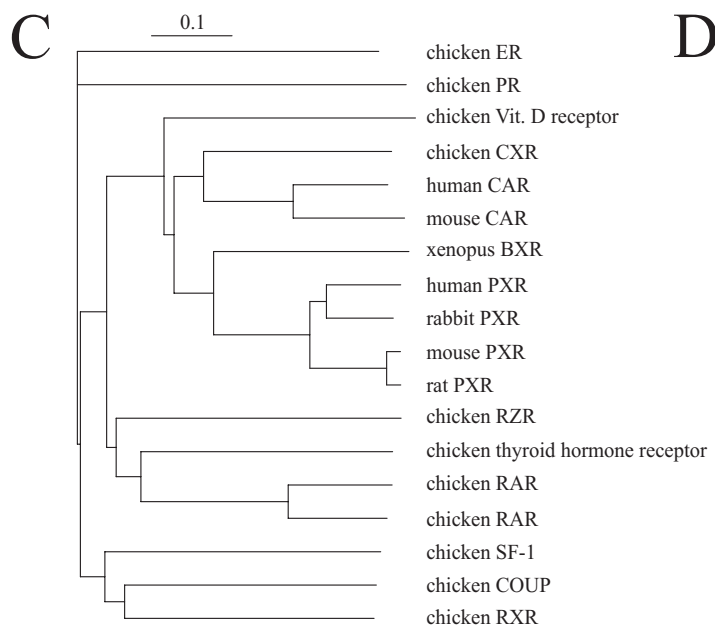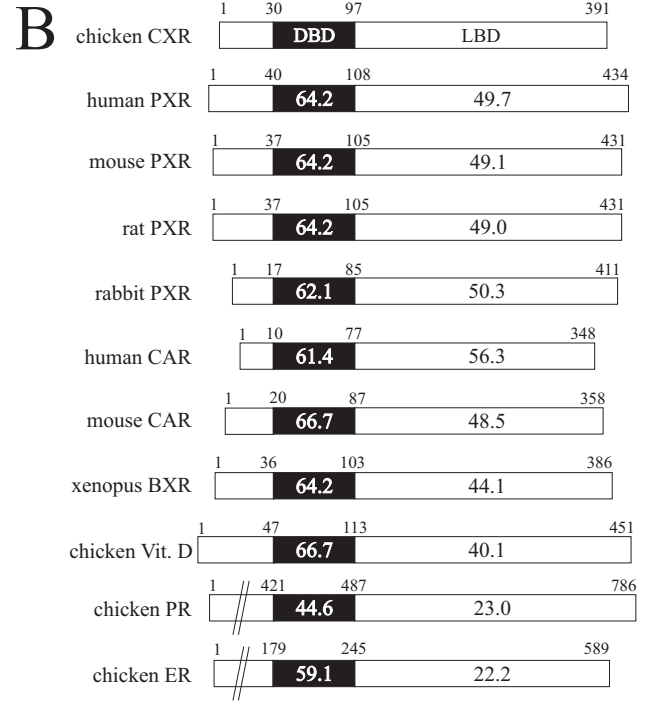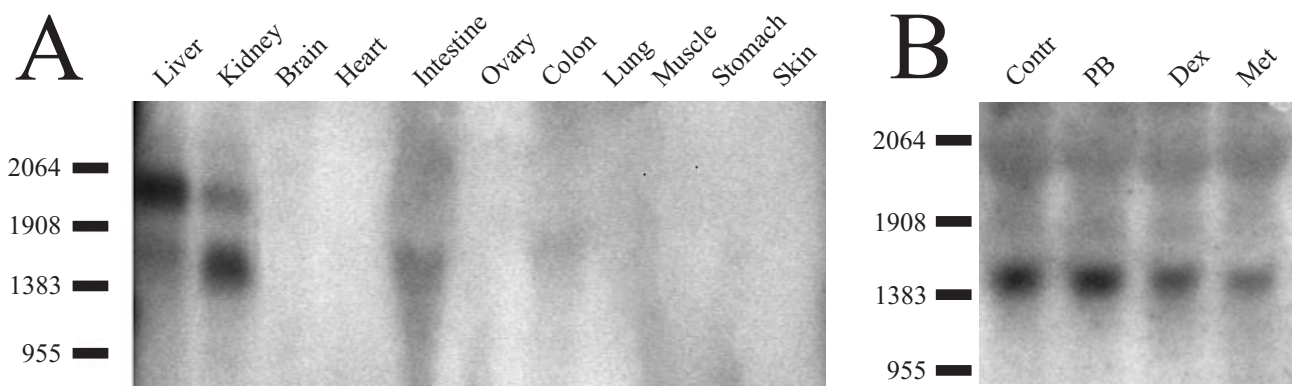
B

C

D

FIG. 2

FIG. 3

A

264-bp PBRU   -1637

TGAACTTCCTTGCCCT
----------------   -1622
ACTTGAAGGAACGGGA

264-bp PBRU
double mutant   -1637

CCGCGGTCCT**GATATC**
----------------   -1622
GGCGCCAGGA**CTATAG**
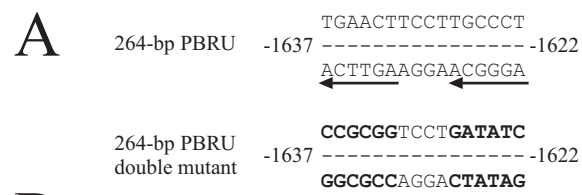
B

	1	2	3	4	5	6	7
[³P]-264-bp PBRU	+	+	+	+	+	+	+
chicken RXR	-	+	-	+	+	+	+
chicken CXR	-	-	+	+	+	+	+
anti-RXR antibody	-	-	-	-	+	-	-
264-bp PBRU	-	-	-	-	-	+	-
264-bp PBRU double mutant	-	-	-	-	-	-	+

a →

b →

c →

FIG. 4

relative CAT level
chicken CXR

relative CAT level
264-bp PBRU

relative mRNA level
CYP2H1 mRNA