RABBIT PREGNANE X RECEPTOR IS ACTIVATED BY RIFAMPICIN

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ABSTRACT:
Reverse transcriptase-polymerase chain reaction was used to amplify a partial cDNA from rabbit lung mRNA that shared 77% protein sequence identity with the mouse pregnane X receptor (PXR). Rapid amplification of cDNA ends from a rabbit kidney cDNA expression library resulted in the isolation of overlapping cDNAs spanning the complete coding sequence. The deduced amino acid sequence of 411 residues exhibited 79% overall amino acid identity with human PXR and 77% identity with mouse PXR. Based on this protein sequence relationship and a similar degree of conservation exhibited by the mouse and human PXR orthologs, the cDNA appears to encode the rabbit PXR ortholog. 5'-rapid amplification of cDNA ends performed on an adaptor-ligated cDNA library from rabbit liver revealed the presence of an alternate mRNA, which differed at the 5'-terminus. RNase protection assays indicated that the alternate mRNA was expressed at >50-fold lower levels in rabbit kidney and liver. Rifampicin treatment of CV-1 cells cotransfected with a rabbit PXR expression plasmid and a luciferase reporter construct containing two copies of the DR3 enhancer from CYP3A23 produced a 6-fold induction of luciferase activity. In contrast, rat PXR was not responsive to this antibiotic under the same conditions. Pregnenolone 16α-carbonitrile was an efficacious activator of rat PXR, but failed to significantly activate rabbit PXR at equivalent concentrations. These results indicate that the ligand activation profile of rabbit PXR is distinct from rat PXR and more closely resembles that of human PXR. The rabbit PXR activation profile is consistent with the cytochrome P450 (P450) 3A6 induction profile in rabbits.

A diverse array of drugs can induce cytochrome P450 (P450) 3A enzymes in rat, rabbit, and human livers, as well as in primary hepatocytes derived from these species; however, these responses are distinct for each species. Hepatic P450 3A enzymes catalyze the metabolism of a large number of clinically administered drugs in humans, and increased expression of these P450s as a result of exposures to inducers can alter human drug metabolism (Guengerich, 1999). A large number of structurally unrelated compounds such as the synthetic glucocorticoid dexamethasone (DEX), the antiluocorticoid pregnenolone 16α-carbonitrile (PCN), phenobarbital, polyhalogenated aromatic hydrocarbons, and the macrolide antibiotics troleandomycin (TAO) and rifampicin (Maurel, 1996) produce distinct P450 3A induction profiles in each species. These species differences confound pharmacological evaluations of drug responses as they limit reliable extrapolation of experimental data from animal model systems to humans. Thus, delineation of the regulatory mechanisms that underlie species differences in P450 3A induction is important for proper evaluation of drug effects and drug-drug interactions that are often associated with the induction of human P450 3A enzymes (Tanaka, 1998). Although the induction of P450 3A in rat liver and rat primary hepatocytes by DEX is suggestive of a role for the glucocorticoid receptor (GR) in the regulation of CYP3A genes, it seemed likely that other regulatory pathways are involved. Unlike a classical GR-mediated response, treatment with either the GR agonist DEX or the GR antagonist PCN results in increased levels of P450 3A mRNA (Burger et al., 1992). In addition, the high pharmacological concentrations of DEX or PCN required for P450 3A induction exceed the concentrations required to induce other GR-regulated proteins (Schuetz et al., 1984). Analysis of the 5′-flanking region of the CYP3A23 gene by in vitro DNA footprinting revealed DEX- and PCN-dependent protein-DNA interactions involving two enhancer elements. Each element is organized as a direct repeat of nuclear receptor sequence motifs, which suggested a role for these receptors in P450 3A induction (Quattrochi et al., 1995; Huss et al., 1996; Huss and Kasper, 1998). These response elements are distinct from GR-binding sites and were not protected in footprinting assays by Escherichia coli-expressed GR (Huss et al., 1996; Huss and Kasper, 1998). These response elements are distinct from GR-binding sites and were not protected in footprinting assays by Escherichia coli-expressed GR (Huss et al., 1996; Huss and Kasper, 1998). These response elements are distinct from GR-binding sites and were not protected in footprinting assays by Escherichia coli-expressed GR (Huss et al., 1996; Huss and Kasper, 1998). These response elements are distinct from GR-binding sites and were not protected in footprinting assays by Escherichia coli-expressed GR (Huss et al., 1996; Huss and Kasper, 1998).
(Pascussi et al., 1999). Transient transfections of rat, rabbit, and human primary hepatocytes with reporter constructs containing these elements demonstrated that species differences in the activation of CYP3A genes by P450 3A inducers is dependent on the species origin of the cellular environment rather than on differences in CYP3A gene structures (Barwick et al., 1996).

The ability of known P450 3A inducers to activate mouse pregnant X receptor (PXR) and increase transcription of a chloramphenicol acetyltransferase reporter through a CYP3A23-DR3 element provided evidence that the activation of CYP3A genes is mediated by PXR (Kliewer et al., 1998). Furthermore, electrophoretic mobility shift assays demonstrated that mouse PXR could bind to a DR3 element, and indicated that this protein–DNA interaction was dependent on heterodimerization with the retinoid X receptor (Kliewer et al., 1998).

Several recent reports have described the human PXR ortholog designated hPXR (Bertilsson et al., 1998), SRX (Blumberg et al., 1998), or hPXR (Lehmann et al., 1998). Also, the rat PXR ortholog has been isolated (Zhang et al., 1999). The DNA binding domains (DBDs) of the human, rat, and mouse PXRs exhibit 92 to 95% protein sequence identity and bind to similar response elements. The ligand-binding domains (LBDs) of the rat and mouse PXRs share 79% identity, whereas the LBDs of the mouse and human PXRs are only 78% homologous and appear to represent pharmacologically distinct receptors as they show differences in ligand activation properties. For instance, mouse PXR is activated by PCN (Kliewer et al., 1998); however, PCN is not an efficacious activator of human PXR (Bertilsson et al., 1998). Also, the human receptor is activated by rifampicin, but this compound does not activate mouse PXR (Lehmann et al., 1998). Similarly, rifampicin is not an efficacious inducer of P450 3A in mouse liver (Wrighton et al., 1985) but it induces P450 3A in human hepatocytes (Kocarek et al., 1995). The activation profiles obtained from the human and mouse PXRs are consistent with the induction of human and rodent P450 3As.

Rifampicin induces P450 3A6 in rabbit liver (Dalet et al., 1986; Potenza et al., 1989) and rabbit hepatocytes (Daujat et al., 1987; Kocarek et al., 1995). Prompted by the discovery of mouse PXR, we developed a PCR strategy for the isolation of PXR-related receptors to explore their role in the induction of P450 3A6 and P450 4A4 in rabbits. P450 4A4 is highly regulated during rabbit pregnancy and, similar to P450 3A, is induced by pharmacological concentrations of DEX and progesterone (Matsubara et al., 1987; Palmer et al., 1993). Here we report the characterization of rabbit PXR and demonstrate that the activation profile is consistent with a role in the mediation of P450 3A6 induction.

**Materials and Methods**

**Chemicals.** Dimethyl sulfoxide, rifampicin, DEX, TAO, clotrimazole, 5β-pregnane-3,20-dione, and farnesol were purchased from Sigma (St. Louis, MO). PCN and RU486 were obtained from Biomol Research Laboratories (Plymouth Meeting, PA).

**Total RNA Isolation and Selection of PolyA+ RNA.** Total RNA was isolated from H4IIEC3 cells using the RNAsig RNA purification kit (Promega, Madison, WI). Tissues were collected from untreated and DEX-administered New Zealand White rabbits as described previously (Palmer et al., 1993) and total RNA was prepared using the RNeasy RNA isolation kit (Qiagen, Valencia, CA). PolyA+ enriched RNA was prepared using oligo-dT cellulose as described (Sambrook et al., 1989). The integrity of total RNA and poly(A)+-enriched RNA was assessed by electrophoresis on denaturing agarose gels containing formaldehyde.

**Isolation of Partial cDNA Clones Encoding Nuclear Receptors.** Multiple protein sequence alignments of mouse PXR with related nuclear receptors revealed a highly conserved peptide sequence, CECKGIPF, in the DBD. This peptide is conserved in most known nuclear receptors. A second peptide, EDQISLLK, is located in the LBD and is conserved in the mouse and human PXRs. This peptide is also conserved in receptors that are more closely related to PXR with the exception of substitutions at one amino acid position, EDQI/S/A/I/T/V/LLK. Mouse PXR-specific primers corresponding to these regions were designed, and low-stringency PCR conditions were applied to amplify PXR-related cDNAs from rabbit lung. The forward primer, PXR165F (5′-TGG GAA GGA TGC AAG GGG TTT TTC-3′) corresponded to nucleotides (nt) 313–336 in mouse PXR (accession no. AF031814) (Kliewer et al., 1998). The reverse primer, PXR798R (5′-CTT CAG CAG GGA GAT CGT GTC CTC-3′) corresponded to nt 949–972 in mouse PXR. Reverse transcription of total RNA (10 μg) isolated from lung tissue of untreated and DEX-administered rabbits (Palmer et al., 1993) was carried out using a 9-mer random primer (Boehringer Mannheim, Indianapolis, IN) and Moloney murine leukemia virus reverse transcriptase (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. The PCR amplification was carried out using the above primers and TurboPfu polymerase for high-fidelity PCR (Stratagene) using the following conditions: 1 denaturation cycle at 94°C for 3 min, followed by 38 cycles of 94°C for 1 min, 56°C for 1.5 min, and 72°C for 1.5 min. The reaction was completed by an additional 10-min extension at 72°C. To assess differentially displayed amplified cDNA species, the reaction products were separated on 1.7% agarose gels and visualized by SYBR green I staining (FMC Bioproducts, Rockland, ME). PCR products of the expected size were ligated into the EcoR V site of pBluescript KS(+) (Stratagene). Similarly, the primer set was used to isolate rat PXR-related cDNAs using reverse transcribed RNA from H4IIEC3 cells. Positive clones were selected after restriction endonuclease digestion, electrophoretic separation on agarose gels, transfer to nitrocellulose membranes, and hybridization to a 32P-labeled mouse PXR cDNA probe under low-stringency hybridization conditions (Sambrook et al., 1989). The mouse PXR probe was produced by PCR using the same primer set on a pGEM plasmid harboring the mouse PXR cDNA and corresponded to nt 313–972 (Kliewer et al., 1998). Nucleotide sequence was obtained from hybridization-positive clones and subjected to database searches using the blast network server at the National Center for Biotechnology and Information.

**Rapid Amplification of cDNA Ends (RACE) from Rabbit Kidney and Liver.** Additional reverse transcriptase-polymerase chain reaction (RT-PCR) amplifications from RNA of various rabbit tissues using internal primers to the partial cDNA clone isolated from rabbit lung indicated that the highest expression was in kidney and liver. Therefore, we selected a AZAP expression library constructed from rabbit kidney RNA and an adaptor-ligated liver cDNA library for RACE. A combination of AZAP arm- and gene-specific primers was used for the RACE reactions. For 5′-RACE, the forward vector primer, SK382 (5′-GCT TTT CCC AGT CAC GAC GTT G)−3′) corresponded to nt 582–603 in pBluescript SK(−) (accession no. X52324). The reverse vector primer, SK825 (5′-GAG GAG ATG ATA ACC TAT CAC AGG)−3′) corresponded to nt 825–848 in pBluescript SK(−). These sequences flank the multiple cloning sites in the AZAP vector. As the library construction was not directional, each of these vector primers was used in combination with the mouse PXR specific primer PXR798R. An aliquot of the PCR reaction served as template for the first nested PCR using each SK primer with the nested reverse primer PR2R (5′-ACT TCT GTG ATG TGG AGG CGG-3′) that corresponded to nt 864–883 of rabbit PXR. The second nested PCR was carried out with reverse primer PR1R (5′-CGA TTA TCA TCC GCT GCT CTC CC-3′) that corresponded to nt 619–641 of rabbit PXR.

For 3′-RACE, PCR amplifications were carried out using each of the vector SK primers in combination with the forward gene-specific primer PXR165F. The nested PCR amplifications were accomplished using each SK primer with the forward primer PR1F (5′-CGG GAG AGC AGG TGA TAA TCC-3′) that corresponded to nt 618–640, and the nested forward primer PR2F (5′-TCC GGC CCC AGC TGA CAG AA-3′) that corresponded to nt 862–881 of rabbit PXR. The following cycling conditions were applied: 1 cycle of denaturation at 94°C for 3 min, followed by 28 cycles of 94°C for 1 min, 57°C for 1.5 min, and 72°C for 3 min. Reactions were completed by an additional incubation at 72°C for 7 min. For 3′-RACE, the combination of SK582 and forward gene-specific primers resulted in the amplification of a significant amount of product of approximately 900 base pairs (bp). The SK582 primer in combination with the reverse gene-specific primers yielded PCR product of
approximately 950 bp from 5'-RACE reactions. PCR products were ligated into pBluescript KS(+) and sequenced.

A consensus sequence was generated for the complete cDNA from multiple 5'- and 3'-RACE cDNA clones with an overlap of 220 nucleotides on the 5'-end and 136 nucleotides on the 3'-end of the original partial rabbit PXR cDNA. To amplify the complete cDNA, new primers were synthesized. The forward KpnI primer (5'-CGGGGATTACCTCGGTAGCGAGTCGTGTTTCGTA-3') corresponded to nt 862–888 of rabbit PXR, used for PCR amplification. These primers were used to isolate the complete coding sequence from the kidney library and also from reverse transcribed kidney RNA. These full-length clones were sequenced and compared with the previously obtained consensus sequence.

For RACE from rabbit liver, poly(A)+-enriched RNA (1 µg) was used for the construction of an adaptor-ligated cDNA library using the Marathon cDNA amplification kit according to instructions supplied by the manufacturer (Clontech, Palo Alto, CA). For 5'-RACE, the adaptor primer (Clontech) and the gene-specific primer, PR2R-27 mer (5'-TTCCAGTCTGTCCGCTTGGG-3'), corresponding to nt 862–888 of rabbit PXR, were used for the first PCR amplification. The nested adaptor primer and a gene-specific PR1R-27 mer (5'-TCCAGTCTGTCCGCTTGGG-3') corresponding to nt 619–645 of rabbit PXR were used to generate nested PCR products. Touch-down PCR was carried out with 1 cycle of denaturation at 94°C for 30 s, followed by 5 cycles at 94°C for 5 s and 70°C for 4 min; 5 cycles at 94°C for 5 s and 68°C for 4 min, followed by 30 cycles at 94°C for 5 s and 66°C for 4 min. The gene-specific primers in these reactions corresponded to primers PR2R and PR1R, with primer length modifications to accommodate the high annealing temperatures necessary for touch-down PCR. Products were ligated into pBluescript KS(+) and sequenced.

Cloning of Rat PXR from H4IIEC3 Cells. A primer set corresponding to nt 151–178 of mouse PXR (PRXSacI, 5'-GGTCAGCTATGCAGCAGGAGGGTGTCGAGCCG-3') and nt 1420–1446 (PRXXhoI, 5'-CGCGGATCTTGGATGAGTGGATGGGGG-3') was used for RT-PCR, using RNA from the rat hepatoma H4IIEC3 cell line. These primers contained additional nucleotides at the 5'-end (underlined) corresponding to the restriction sites used for directional cloning. PCR was carried out with 1 cycle of denaturation at 94°C for 3 min, followed by 38 cycles of 94°C for 1 min, 58°C for 1.5 min, and 72°C for 3 min. The reactions were completed by an additional incubation at 72°C for 10 min. The resulting PCR products were ligated into pBluescript KS(+) and sequenced.

RNase Protection Assays. Complementary riboprobes for rabbit PXR were generated from linearized plasmids using the RNA transcription kit according to the protocol of the manufacturer (Stratagene). The examination of relative expression levels of alternate mRNAs used two probes. A cDNA fragment encoding riboprobe 1, which corresponds to nt 1–641 in the rabbit PXR sequence, was inserted into the EcoRI site of pBluescript KS(+), and this plasmid was linearized with HindIII. Probe 1 was expected to result in a 641-bp fragment for complete protection (mRNA1) and a 472-bp fragment representing the alternate mRNA (mRNA2). Riboprobe 2 consisted of a segment of 290 nt, with nt 160–290 of rabbit PXR being sequence common to both mRNAs and the additional 118 nt upstream of position 169 containing divergent sequence between the two mRNAs. Two protected fragments of 290 bp (mRNA2) and 172 bp (mRNA1) were predicted with probe 2. For examination of tissue distribution, the initial partial rabbit PXR cDNA was used as the riboprobe (probe 3). This was ligated into the EcoRV site of pBluescript KS(+) and linearized by XbaI before transcription. This probe spanned nt 359-1017 of rabbit PXR and was expected to result in a completely protected fragment of 658 bp. Riboprobes were generated by transcription from the linearized plasmids in the presence of [α-32P]UTP (800 Ci/mmole) using T7 RNA polymerase for riboprobes 1 and 2, and T3 RNA polymerase for riboprobe 3. After transcription, the probes were purified on 6% acrylamide gels containing 6 M urea. For examination of tissue distribution, 106 cpm were used for hybridization to 40 µg of total RNA. Total RNA (120 µg) or mRNA (10 µg) was used to investigate the levels of alternative rabbit PXR transcripts. The RPAII kit (Ambion, Austin, TX) was used for hybridization of probes and RNase digestions. After RNase digestion, protected fragments were separated on sequencing gels. Two 32P-labeled molecular size markers were included, a 1-kb DNA ladder (Life Technologies, Grand Island, NY), and the φx174RF ladder (NEB, Beverly, MA). Gels were analyzed with a Molecular Dynamics PhosphorImager, model SI (Sunnyvale, CA).

Construction of Expression Vectors and Transient Transfection Studies. Three expression constructs designated rabbit PXR, rabbit PXR-CTG, and rabbit PXR-ATG were generated to evaluate potential differences in expression resulting from the use of alternate translation start sites. The rabbit-PXR construct contained the complete coding sequence of mRNA1 that had been amplified in its entirety with the KpnI and XbaI primers and inserted into the KpnI and XbaI sites of pCMV5. This coding sequence contained the CTG corresponding to the putative translation start site of human PXR, and the downstream ATG encoding the first in-frame methionine residue. The rabbit PXR-CTG construct contained the CTG corresponding to the translation start site of human PXR, but carried a mutation that changed the codon of the first in-frame methionine to ATC, which encodes an ileucine. The mutant was generated by using the Quick Change Site Directed Mutagenesis method with complementary primers corresponding to nt 245–273 of rabbit PXR (forward PR5-GAA GCA GAC TAC ATG GGT GCA AGG CCC AC, the mutated nucleotide is underlined) according to the manufacturer’s instructions (Stratagene). The CTG codon corresponding to the translation start site of human PXR was deleted in the rabbit PXR-ATG construct. The deletion mutant was generated by PCR using a forward primer containing a 5'- EcoRI cloning site. This primer corresponded to nt 251–274 of the rabbit PXR cDNA and was used in combination with a reverse primer that corresponded to nt 359–382. The PCR product was subjected to consecutive BstEI/EcoRI digestion before insertion into the BstEI and EcoRI digested rabbit PXR cDNA. Each of these expression vectors was sequenced to verify its integrity. The rat PXR cDNA was subcloned into the EcoRI and SalI sites of pCMV5. The luciferase reporter plasmid pLUC-(DR3), was generated by insertion of two copies of the CYP3A23-DR3 element into the luciferase reporter vector containing the Herpes simplex virus thymidine kinase promoter. The β-galactosidase expression construct pCMV/βGal was purchased from CLONTECH (Palo Alto, CA). For transient transfection studies, CV-1 cells were maintained in Dulbecco’s modified Eagle’s medium containing high glucose (Life Technologies, Frederick, MD) supplemented with 10% fetal calf serum (Summit, Ft. Collins, CO). Transfection experiments were carried out using methods described previously (Palmer et al., 1995; Hsu et al., 1998). Briefly, the constructs were introduced into CV-1 cells by a modified calcium phosphate coprecipitation procedure and exposed to DNA for 16 h. Subsequently, the cells were washed with serum-free medium and then placed in medium containing 10% charcoal/dextran-treated fetal bovine serum (Hyclone, Logan, UT) and the various test compounds. Stock solutions of the test compounds were prepared in dimethyl sulfoxide (DMSO) and the final concentration of the solvent was 0.25% (v/v). The medium that contained test compound was replaced by fresh medium after 24 h and cultures were continued for an additional 24 h. Cells were harvested in Dulbecco’s phosphate-buffered saline without calcium and magnesium and resuspended in lysis buffer containing 100 mM potassium phosphate buffer, pH 7.8, containing 1 mM dithiothreitol and 0.05% Triton X-100. The harvested cells were lysed by three freeze/thaw cycles of alternate immersion in liquid nitrogen and water. After centrifugation, luciferase activity was measured in cytosolic fractions using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI). β-galactosidase activity was assayed with the Galacto-Light Kit (Tropix, Bedford, MA) and used to normalize luciferase values for the same preparation of lysate.

Results

A report indicating that PXR is activated by progesterone and its metabolites (Kliwer et al., 1998) led us to investigate whether a rabbit PXR-related receptor, possibly a paralog, mediates the elevated expression of P450 3A4 during pregnancy as well as the induction of this enzyme by pharmacologic concentrations of progesterone or DEX (Matsubara et al., 1987; Palmer et al., 1993). We also sought to isolate cDNAs encoding the rabbit PXR ortholog to clarify the role of this receptor in P450 3A6 induction.

In anticipation of PXR sequence divergence among species and to cast a broader net for the isolation of PXR paralogs, multiple protein sequence alignments were used to aid in the selection of primers for
A sequence alignment of mouse PXR and related nuclear receptors revealed two regions of significant identity (bold). The highly conserved peptide CEGCKGF is located in the LBD and is conserved in receptors that are more closely related to PXR. These regions were selected to design forward and reverse mouse PXR-specific oligonucleotide primers. Application of this primer set on reverse transcribed RNA from rabbit lung resulted in a partial cDNA of approximately 600 nt.

PCR amplification. The alignment of mouse PXR with related nuclear receptors revealed two regions of high conservation (Fig. 1). The first peptide, CEGCKGF, is located within the conserved part of the DBD and is shared by most nuclear receptors. The second peptide, EDQISLLKK, is located in the C-terminal LBD of mouse PXR. This peptide is conserved in other receptors that are closely related to PXR, except for substitutions at a single position, EDQI(S/A/I/T/V/LLKK) is located in the LBD and is conserved in receptors that are more closely related to PXR. These regions were selected to design forward and reverse mouse PXR-specific oligonucleotide primers. Application of this primer set on reverse transcribed RNA from rabbit lung resulted in a partial cDNA of approximately 600 nt.

Analysis of the rabbit PXR nucleotide sequence revealed an open reading frame beginning at nucleotide 113 (Fig. 3). The first in frame initiation codon, ATG, is found at nucleotide 257 and is surrounded by a T at -3 and a G at +4 (A in ATG = +1). This does not correspond to an optimal translation initiation context that is characterized by a purine residue (A/G) at -3 and a G at position +4 (Kozak, 1991). The deduced amino acid sequence of 411 residues arising from the use of this initiation codon exhibits 79 and 77% overall protein sequence identity with human and mouse PXRs, respectively. The human PXR cDNA does not exhibit a methionine initiation codon, but rather appears to use a CTG codon for leucine in a consensus context for initiation. This CTG codon and a consensus context for initiation are conserved in the rabbit sequence upstream of the initiation codon (Fig. 3).

Protein sequence alignments of mouse PXR with related nuclear receptors.

A sequence alignment of mouse PXR and related nuclear receptors revealed two regions of high conservation (bold). The highly conserved peptide CEGCKGF is located in the LBD and is conserved in receptors that are more closely related to PXR. These regions were selected to design forward and reverse mouse PXR-specific oligonucleotide primers. Application of this primer set on reverse transcribed RNA from rabbit lung resulted in a partial cDNA of approximately 600 nt.

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The DBDs of the rat, mouse, rabbit, and human PXRs are highly conserved and share 92 to 95% protein sequence identity. This is consistent with the ability of these receptors to recognize and stimulate transcription through the same response elements. In contrast, the DBDs of these receptors display a considerable degree of divergence. The LBD protein sequence identities for rodent, rabbit, and human PXRs are summarized in Table 1. Rabbit PXR shares 82% amino acid identity with human PXR, 78% with mouse PXR, and 77% with rat PXR. Rat and human PXRs share 76% sequence identity, whereas mouse PXR and human PXR exhibit 78% amino acid sequence identity. In contrast, there is 97% protein sequence identity in this region between the mouse and rat PXRs. The degree of LBD divergence between mouse and rabbit, mouse and human, or rabbit and human PXR LBDs is unusual for receptor orthologs and suggests that these receptors may be paralogs.
5′-RACE experiments carried out with the adaptor-ligated liver cDNA library also resulted in the isolation of cDNA fragments exhibiting 118 nucleotides of divergent sequence at the 5′ end (mRNA2) relative to the first cDNA (mRNA1) as shown in Fig. 3. The open reading frame for the coding sequence extends across the length of this alternative sequence, indicating that mRNA2 would exhibit a longer N-terminal sequence. Interestingly, Bertilsson et al. (1998) have reported an alternative cDNA for human PXR that encodes an alternative N terminus, and the sequence divergence occurs at the same position seen for the rabbit cDNAs. To confirm the expression of mRNA2 and to analyze the relative expression levels of the alternative rabbit mRNAs, RNase protection assays were performed using RNA from rabbit liver and kidney with riboprobes specific for each mRNA (Fig. 4). When a riboprobe corresponding to mRNA1 was used (probe 1), Fig. 4A, a prominent protected RNA species was detected in rabbit liver and kidney at approximately 640 bp (Fig. 4B), which corresponds to the size predicted for complete protection by mRNA1. A partially protected fragment corresponding to mRNA2 was not clearly evident although this could reflect a relatively high background (Fig. 4B). Riboprobe 2, corresponding to mRNA2, yielded a major partially protected fragment in liver and kidney RNA of 172 bp that corresponds to the predicted size of the sequence that is shared by both mRNAs. In addition, a very low-abundance, protected RNA species at 290 bp that reflects complete protection of probe 2 was evident. Quantification of the partially protected 172-bp fragment arising from protection by mRNA1 and the 290-bp fragment representing mRNA2 indicates that mRNA1 is expressed at 50-fold higher levels relative to mRNA2. The similar expression patterns obtained from kidney and liver indicate that the relative abundance of these mRNAs appears to be equivalent in both tissues.

RNase protection assays were also used to examine the relative abundance of PXR mRNA in different rabbit tissues. Detectable levels of mRNA were observed in liver, kidney, and small intestine as evidenced by the protected fragment of approximately 650 bp (Fig. 5). This is consistent with the tissue distribution pattern reported for mouse PXR (Kliewer et al., 1998). Although the partial cDNA clone was isolated by RT-PCR from lung mRNA prepared from DEX-treated rabbit, specific mRNA in lung and other tissues examined was not apparent, suggesting that mRNA levels were below the detection limit of the experimental conditions used for the assay.

The initial partial cDNA clone encoding rabbit PXR was isolated from a PCR product obtained from lung RNA of a DEX-treated rabbit by using a primer set that was designed to cast a broad net for PXR-related receptors. This PCR product appeared to be more abundant than the PCR product obtained from lung RNA of untreated rabbits, suggesting the possibility that DEX treatment might increase PXR mRNA levels in rabbits.

**Fig. 3.** Nucleotide and deduced amino acid sequence of rabbit PXR.

The nucleotide sequence is a composite of the initial partial cDNA clone and overlapping RACE clones. The nucleotide sequence (bold) represents cDNA corresponding to mRNA1. The sequence shown in italics corresponds to the alternate 5′-terminus of mRNA2. Both mRNA sequences are identical downstream of nucleotide 168 in mRNA1. Boxed codons indicate the first in frame CTG and ATG encoding the putative translation start sites discussed in the text. ▽ depict the beginning and the end of the rabbit PXR cDNA that was amplified in its entirety and used for expression in transient transfection assays. □ depict stop codons.

**TABLE 1**

Amino acid sequence identity for ligand binding domains of PXR orthologs

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PXR using RT-PCR reactions confirmed the presence of the rabbit PXR cDNA in the lung but did not provide evidence for induction by DEX. However, the abundance of this mRNA was below the detection limit of the RNase protection assay conditions and remained undetectable in lung mRNA prepared from either untreated or DEX-treated rabbits. To determine the potential inducibility of rabbit PXR mRNA by DEX, we examined the level of mRNA in tissues that exhibit detectable mRNA levels. In addition, a recent report demonstrated the presence of the rabbit PXR cDNA encoding the amino terminus (Fig. 6A) and examined the translational activation of a cotransfected luciferase reporter that uses a thymidine kinase promotor and two copies of the CYP3A23-3A6 in rabbits, and this compound was selected to examine the transcriptional activation of a cotransfected luciferase reporter that uses a thymidine kinase promotor and two copies of the CYP3A23-(DR3) PXR response element. The rabbit PXR construct containing both initiation codons produced an approximately 4- to 5-fold increase in luciferase expression after treatment with rifampicin (Fig. 6B). The ability of the CTG to initiate translation was investigated by mutation of the downstream ATG to an ATC, which substitutes an isoleucine residue for the methionine residue (rabbit PXR-CTG). The cotransfection of CV-1 cells with the reporter and this construct resulted in luciferase activity similar to that obtained with rabbit PXR. This indicates that the methionine codon is not required for initiation of translation (Fig. 6B). The elimination of the CTG and adjacent upstream sequence (rabbit PXR-ATG) also did not result in significantly diminished reporter activity, as the transcriptional activation seen with this con-

**Fig. 4. Two alternate transcripts exist for rabbit PXR that differ in their 5'-termini.**

A, diagram illustrates mRNA1- and mRNA2-specific riboprobes and their predicted protection patterns. Probe 1 represents the 5'-RACE clone isolated from the AZAP kidney expression library (mRNA1) and probe 2 represents the 5'-RACE clone isolated from the adaptor-ligated liver cDNA library (mRNA2). Probe 1 corresponded to nt 1–641 in the rabbit PXR sequence (720 nt, including vector sequence) and was predicted to result in a fragment of 641 bp reflecting complete protection by mRNA1 and a fragment of 472 bp for partial protection by mRNA2. Probe 2 (360 nt, including vector sequence) was predicted to yield a segment of 290 bp corresponding to full protection of mRNA2 and a 172 bp fragment for partial protection by mRNA1. The vector sequence is indicated by a line, the shared sequence by black, the white boxes represent mRNA1 specific sequence, and the gray boxes indicate mRNA2 specific sequence. The coding region is depicted by the characteristic consensus sequence for translation initiation. In contrast, the first in frame ATG, which is the only in frame methionine codon before the DBD, does not have an optimal context for initiation of translation. Thus, rabbit PXR exhibits two putative initiation start sites and either could potentially serve as the initiation site for translation. To test these putative initiation codons independently, we introduced either a mutation or a deletion into the 5'-end of the rabbit PXR cDNA encoding the amino terminus (Fig. 6A) and examined the consequence of these modifications in transient transfection assays of CV-1 cells. The antibiotic rifampicin has been shown to induce P450 3A6 in rabbits, and this compound was selected to examine the transcriptional activation of a cotransfected luciferase reporter that uses a thymidine kinase promotor and two copies of the CYP3A23-(DR3) PXR response element. The rabbit PXR construct containing both initiation codons produced an approximately 4- to 5-fold increase in luciferase expression after treatment with rifampicin (Fig. 6B). The ability of the CTG to initiate translation was investigated by mutation of the downstream ATG to an ATC, which substitutes an isoleucine residue for the methionine residue (rabbit PXR-CTG). The cotransfection of CV-1 cells with the reporter and this construct resulted in luciferase activity similar to that obtained with rabbit PXR. This indicates that the methionine codon is not required for initiation of translation (Fig. 6B). The elimination of the CTG and adjacent upstream sequence (rabbit PXR-ATG) also did not result in significantly diminished reporter activity, as the transcriptional activation seen with this con-

**Fig. 5. Rabbit PXR tissue distribution.**

Total RNA (40 µg) from various rabbit tissues was hybridized to a riboprobe generated from the partial cDNA for rabbit PXR and used in a RNase protection assay as described in Materials and Methods. This riboprobe (probe 3) corresponds to nt 359-1017 in the rabbit PXR nucleotide sequence and does not discriminate between mRNA1 and mRNA2. The probe was expected to yield a protected fragment of 658 bp (arrow). The tissue source of RNA is indicated above the respective lane: MW lane, 6x174RF DNA ladder; yRNA, 40 µg of yeast RNA hybridized to probe (10⁵ cpm) followed by RNase treatment; probe, undigested probe (500 cpm); other lanes, 40 µg RNA from the indicated source was hybridized to probe (10⁵ cpm) and subjected to RNase treatment.

The cloning of human PXR (hPAR1) (Bertilsson et al., 1998) and SXR (Blumberg et al., 1998) revealed a nonconventional translation initiation site from a CTG encoding a leucine residue. This CTG codon is flanked by a consensu sequence for initiation of translation as characterized by an A at the −3 position and a G at the +4 position (Kozak, 1991). The rabbit PXR nucleotide sequence also contains an in frame CTG 63 nucleotides upstream of the first ATG, which is present in both rabbit PXR mRNAs (Fig. 3). This CTG is at a similar position relative to the human sequence and is also surrounded by a characteristic consensus sequence for translation initiation. In contrast, the first in frame ATG, which is the only in frame methionine codon before the DBD, does not have an optimal context for initiation of translation. Thus, rabbit PXR exhibits two putative initiation start sites and either could potentially serve as the initiation site for translation. To test these putative initiation codons independently, we introduced either a mutation or a deletion into the 5'-end of the rabbit PXR cDNA encoding the amino terminus (Fig. 6A) and examined the consequence of these modifications in transient transfection assays of CV-1 cells. The antibiotic rifampicin has been shown to induce P450 3A6 in rabbits, and this compound was selected to examine the transcriptional activation of a cotransfected luciferase reporter that uses a thymidine kinase promotor and two copies of the CYP3A23-(DR3) PXR response element. The rabbit PXR construct containing both initiation codons produced an approximately 4- to 5-fold increase in luciferase expression after treatment with rifampicin (Fig. 6B). The ability of the CTG to initiate translation was investigated by mutation of the downstream ATG to an ATC, which substitutes an isoleucine residue for the methionine residue (rabbit PXR-CTG). The cotransfection of CV-1 cells with the reporter and this construct resulted in luciferase activity similar to that obtained with rabbit PXR. This indicates that the methionine codon is not required for initiation of translation (Fig. 6B). The elimination of the CTG and adjacent upstream sequence (rabbit PXR-ATG) also did not result in significantly diminished reporter activity, as the transcriptional activation seen with this con-
fect pCMV activity. The bar graph represents luciferase values that were normalized to cotransfected with a CYP3A23-(DR3)2-TK luciferase reporter distinct proline residue. When the rabbit or rat PXR expression vectors were the clone that was used in this study encodes a histidine rather than a amino acid difference is seen in the hinge region at residue 135, where study diverges at amino acids in the N terminus due to the use of a cell line H4IIC3. The cloning and characterization of a rat PXR cDNA constructed using a cDNA cloned by RT-PCR from the rat hepatoma these experiments. For comparison, a rat PXR expression vector was construct containing both potential translation start sites was used in these experiments. For comparison, a rat PXR expression vector was constructed using a cDNA cloned by RT-PCR from the rat hepatoma cell line H4IIC3. The cloning and characterization of a rat PXR cDNA was reported recently (Zhang et al., 1999), and the clone used in this study diverges at amino acids in the N terminus due to the use of a PCR primer corresponding to the mouse sequence. One additional amino acid difference is seen in the hinge region at residue 135, where the clone that was used in this study encodes a histidine rather than a proline residue. When the rabbit or rat PXR expression vectors were cotransfected with a CYP3A23-(DR3)2-TK luciferase reporter distinct activation profiles were seen at equivalent concentrations for several test compounds including rifampicin, PCN, clotrimazole, RU486, and 5-β-pregnane-3, 20-dione (Fig. 7). Rifampicin elicited a >3-fold induction of the reporter in the presence of rabbit PXR, but induction by rifampicin at equivalent concentrations was not evident when rat PXR was cotransfected. In contrast, PCN activated luciferase expression approximately 6-fold over DMSO-treated cells when rat PXR was cotransfected, whereas the efficacy of PCN to stimulate reporter activity was very low by rabbit PXR under the same conditions. Examination of higher concentrations indicated that 100 μM PCN elicited a response from rabbit PXR; however, the stimulation seen was half of that exhibited by rat PXR at these concentrations (data not shown). The antifungal drug clotrimazole, which is a modest inducer of P450 3A6 in rabbits (Kocarek et al., 1995) produced a 2-fold increase of reporter activity by rabbit PXR over the DMSO control (P < .001). Equivalent concentrations of this compound did not produce a significant activation by rat PXR. The GR and progesterone receptor antagonist RU486 elicited a modest response with rabbit PXR, whereas a 3- to 4-fold increase in luciferase activity was measured for rat PXR under the same conditions. Similarly, the progesterone metabolite 5-β-pregnane-3, 20-dione, which is a strong activator of human PXR (Bertilsson et al., 1998), was not an efficacious activator of rabbit PXR, but generated about a 4-fold increase in reporter activity in the presence of rat PXR at equivalent concentrations. DEX, used at 50 μM, produced an approximate 9-fold (≥3.9) increase of reporter activity with rat PXR and a 5-fold (±1.5) increase with rabbit PXR (data not shown). Both receptors also responded similarly to farnesol, which produced an approximately a 3-fold induction of reporter expression. TAO, which is an inducer of P450 3A6 did not elicit statistically significant reporter activation by either receptor relative to DMSO treatment at 100 μM concentrations (data not shown).

Fig. 6. Analysis of alternate translation initiation codons.

A, schematic representation of the expression vectors rabbit PXR, rabbit PXR-CTG, and rabbit PXR-ATG. The rabbit PXR plasmid contained both the CTG codon corresponding to that used by human PXR and the first in frame ATG codon. The relative position of each codon is indicated by arrows, and the open reading frame is depicted by the open bar. The rabbit PXR-CTG construct contained the CTG and carried a single nucleotide mutation that changed the ATG encoding the first in frame methionine to an ATC, which substitutes an isoleucine residue. In the rabbit PXR-ATG construct, the immediate context of the methionine codon was retained but the region upstream containing the CTG was deleted. B, CV-1 cells were cotransfected with each expression construct and a Herpes Simplex Virus thymidine kinase promoter driven luciferase reporter containing two copies of the CYP3A23-(DR3)2 response element. Cells were treated with vehicle (DMSO) or rifampicin for 48 h as described in Material and Methods and assayed for luciferase reporter activity. The bar graph represents luciferase values that were normalized to cotransfected pCMVβGAL and the data is expressed as fold induction of reporter activity by rifampicin (closed bars, 100 μM) relative to vehicle treated cells (DMSO, open bars). The data reported are from transfections and treatments carried out in triplicate. The error bars indicate the S.D. from the mean. Similar results were obtained from an additional experiment performed in triplicate.

Fig. 7. Differential ligand activation profiles of rabbit and rat PXRs.

CV-1 cells were cotransfected with an expression vector for either rabbit PXR (closed bars), or rat PXR (open bars) and a CYP3A23-(DR3)2-TK luciferase reporter. Following a 16-h incubation with DNA, the cells were treated with vehicle (DMSO) or test compound for 48 h and assayed for luciferase reporter activity. The bar graph represents luciferase values that were normalized to cotransfected pCMVβGAL and expressed as fold induction of reporter activity by the tested compound relative to solvent-treated cells. Rifampicin (RIF, 100 μM), PCN (10 μM), clotrimazole (CLT, 10 μM), RU486 (10 μM), 5 β-pregnane-3, 20-dione (5B, 10 μM). The data reported are the mean values from a minimum of two experiments with transfections and treatments carried out in triplicate. The error bars indicate the S.D. from the mean for the pooled observations. The stars denote statistical significance (P < .001) as determined by Student’s t test.
Distinct activation profiles are exhibited by rabbit and rat PXR at equivalent concentrations of the tested compounds. Rabbit PXR is selectively activated by rifampicin, but rifampicin is not an efficacious activator of rat PXR. Rat PXR is selectively activated by PCN and this compound is less efficacious at activating rabbit PXR. Other compounds including RU486 and 5β-pregnane-3, 20-dione activate rat PXR but are less efficacious activators of rabbit PXR at the same concentrations. These differences in activation profiles are consistent with the induction profile of P4503A enzymes in these species and can be attributed to the protein sequence diversity that is seen for the LBDs of these receptors.

While this work was in progress, several other laboratories reported the cloning of a human PXR ortholog that, similar to rabbit PXR, did not respond to PCN but was activated by rifampicin (Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998). These results mirror the responses seen for human P450 3A enzymes in human primary hepatocytes (Kocarek et al., 1995). Thus, the species differences observed in the differential activation of CYP3A genes in rabbits and humans are reflected in the activation profile produced by the corresponding receptor, and this result provides an explanation for the interspecies differences observed for P450 3A induction. These results are also consistent with the observation that the cell environment rather than differences in CYP3A gene response elements governs the pathways that lead to species differences in the differential induction profiles of P450 3A enzymes (Barwick et al., 1996). Although rabbit and human PXR share similar responses to PCN and rifampicin, differences are apparent for 5β-pregnane-3, 20-dione and RU486. Both steroids are activators of human PXR (Bertilsson et al., 1998; Lehmann et al., 1998), but these compounds fail to significantly activate rabbit PXR in transient transfection studies. However, both compounds activate mouse and rat PXR. These results suggest that human PXR shares certain aspects of differential ligand recognition with rabbit and rodent PXR.

Two different mRNAs encoding alternative N termini were evident for rabbit PXR that are likely to arise by differential RNA splicing. Two apparent splice variants with different 5′-termini have also been described for human PXR (Bertilsson et al., 1998). The start of divergent 5′-sequence for human PXR that results in two forms, hPARI and hPAR2, occurs at a similar site in rabbits and gives rise to mRNA1 and mRNA2. The abundance of mRNA1 is >50-fold higher than mRNA2 in either liver or kidney. The functional significance of the alternate rabbit PXR mRNA is unknown.

One of the human splice variants, hPAR2, initiates from an ATG, whereas the other, hPARI or SXR, exhibits a shorter amino terminus that contains a CTG codon in a Kozak sequence context that is thought to initiate the translation of hPARI or SXR (Bertilsson et al., 1998; Blumberg et al., 1998). The predominant rabbit PXR transcript, mRNA1, also exhibits an in-frame CTG codon that has a similar context and that is at a similar position as that found in hPARI. This initiating leucine is located 20 residues upstream of the first in frame methionine. Thus, two putative initiation start sites are present in the rabbit PXR mRNA and either one could serve as the initiation of translation. To assess the role of these putative initiation codons for the start of translation, we constructed expression vectors that contained either the CTG without the downstream ATG or the ATG lacking the upstream residues. These constructs retained the native context for the two codons and were examined for their ability to express PXR and activate the luciferase reporter in CV-1 cells. The presence of either initiation codon generated sufficient functional protein to activate the reporter, which suggests that both the ATG and CTG can be used for initiation of translation. It is unclear if the
additional N-terminal sequence in the CTG translation product could affect protein processing, turnover, or other receptor functions.

The cloning and characterization of PXR from different species has demonstrated clearly that the species differences seen for P450 3A induction can be accounted for by the action of a single receptor. Other nuclear receptors have been implicated in other P450 induction pathways and are likely to also play a role in species-specific drug responses. For example, peroxisome proliferators elicit hepatomegaly and liver carcinomas in mice, but humans are refractory to these effects (Peters et al., 1997). Targeted disruption of the peroxisome proliferator-activated receptor PPARα (Lee et al., 1995) eliminated the adverse effects of peroxisome proliferators and prevented development of carcinomas. PPARα mediates P450 4A induction as well as other peroxisomal enzymes and is expressed at approximately 10-fold lower levels in human liver compared with mouse liver (Palmer et al., 1998). These quantitative differences in receptor expression are likely to contribute to the response differences seen between mice and humans. Another example is provided by the constitutively active nuclear receptor CAR, which has been identified as modulator of CYP2B gene expression in response to phenobarbital (Honkakoski et al., 1998; Sueyoshi et al., 1999). Similar to PXR, an unusually high degree of divergence is seen for the LBDs of human (Baes et al., 1994) and mouse CAR (Choi et al., 1997) that is likely to contribute to species differences in the ligand activation profiles of these receptors.

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References


