

Induction of Rat Organic Anion Transporting Polypeptide 2 by Pregnenolone-16 α -carbonitrile Is via Interaction with Pregnane X Receptor

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ABSTRACT

The rat organic anion transporting polypeptide 2 (oatp2; Slc21a5) is a liver transporter that mediates the uptake of a variety of structurally diverse compounds, and has a high affinity for cardiac glycosides. Treatment of rats with pregnenolone-16 α -carbonitrile (PCN), a ligand for the rodent pregnane X receptor (PXR), significantly enhances the rat oatp2 gene expression. To understand the molecular mechanism of oatp2 induction by PCN, rat oatp2 gene was cloned. The rat oatp2 gene consists of 16 exons; alternative splicing of the second noncoding exon gives rise to the two published rat oatp2 cDNAs. Approximately 8700 base pairs (bp) of the 5'-flanking region of the rat oatp2 gene were linked to the luciferase reporter gene and used in transient transfection assays in H4IIE cells. Treatment of PCN induced the expression of the reporter gene in a dose-dependent manner. Four potential PXR response elements (PXREs) were identified in the 5'-flanking

region of the rat oatp2 gene. One element (DR3-1) is located approximately –5000 bp with three more (DR3-2, DR3-3, and DR3-4) clustered at about –8000 bp. Results from electrophoretic mobility shift assays showed that the PXR-retinoid X receptor α heterodimer binds to the DR3-2 with the highest affinity, to the DR3-4 and DR3-1 with a lower affinity, and weakly or not at all to the DR3-3. Furthermore, a series of partial deletions of the 5'-flanking region illustrated that both the proximal and distal clusters of PXREs are required for maximal induction of rat oatp2 by PCN. In conclusion, these data elucidate the molecular mechanism by which PCN treatment induces rat oatp2 gene expression. In addition, this study identifies rat oatp2 as a direct PXR-targeted gene and further supports the hypothesis that activation of PXR affects a network of genes that is involved in either metabolism or transport of drugs, steroids, and bile acids.

Organic anion transporting polypeptide 2 (oatp2; Slc21a5) is a member of the organic anion transporting polypeptide family that mediates sodium- and ATP-independent transport of a variety of structurally unrelated endogenous and exogenous compounds, including conjugated and unconjugated bilirubin, conjugated steroids, neutral compounds, some type II organic cations, thyroid hormones T3 and T4, and bile salts (Reichel et al., 1999; Suzuki and Sugiyama, 2000). Cardiac glycosides, such as digoxin and ouabain, are transported with very high affinity by oatp2 (Noe et al.,

1997). Oatp2 is localized to the hepatic sinusoidal membrane (Noe et al., 1997; Abe et al., 1998, 1999), with selective expression in the midzonal to perivenous hepatocytes (Reichel et al., 1999). Expression of oatp2 has also been detected in brain and retina (Noe et al., 1997; Abe et al., 1998, 1999; Gao et al., 1999).

Research from this laboratory has demonstrated that hepatic uptake of cardiac glycosides increases after treatment with some microsomal enzyme inducers, such as phenobarbital and pregnenolone-16 α -carbonitrile (PCN) (Klaassen and Plaa, 1968; Klaassen, 1970a,b, 1974a,b; Eaton and Klaassen, 1979). However, the mechanism(s) by which this phenomenon occurs is unknown. The advances in cloning of hepatic transporters have made the in-depth investigation of this phenomenon possible. Research from this laboratory demonstrated that the protein and mRNA expression of rat hepatic oatp2 are increased by PCN treatment in adult and

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ABBREVIATIONS: oatp2, organic anion transporting polypeptide 2; PCN, pregnenolone-16 α -carbonitrile; PXR, pregnane X receptor; DR, direct repeat; RXR α , retinoid X receptor α ; MDR1, multidrug resistant protein 1; BAC, bacterial artificial chromosome; bp, base pair(s); UTR, untranslated region; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; PXRE, pregnane X receptor response element; AhR-Arnt, aryl hydrocarbon receptor/aryl hydrocarbon receptor nuclear translocator; HNF, hepatocyte nuclear factor; NF- κ B, nuclear factor- κ B; STAT, signal transducers and activators of transcription.

newborn animals (Klaasen et al., 2000; Rausch-Derra et al., 2001). The induction of *oatp2* by PCN seems to be due to increased transcription of the *oatp2* gene (Guo et al., 2002).

PCN is a synthetic antiglucocorticoid identified by virtue of its ability to induce protection from various forms of intoxicants in rodents (Selye, 1971). Further research revealed that PCN mediated this protection from chemical poisons by inducing drug-metabolizing activities in liver (Kourounakis et al., 1976). It was subsequently discovered that PCN treatment induces the transcription of the CYP3A subfamily of cytochrome P450 monooxygenases, which metabolize over half of the environmental chemicals and clinically prescribed drugs (Quattrochi and Guzelian, 2001). Induction of the rat *CYP3A1* gene by PCN is mediated through activation of the orphan nuclear receptor family member PXR (NR112; Barnouin et al., 1998; Kliewer et al., 1998).

PXR has been cloned in multiple species, including the rat (Zhang et al., 1999), mouse (Kliewer et al., 1998), rabbit (Savas et al., 2000), and human (Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998), in which PXR is also called the steroid and xenobiotic receptor. PXR and *CYP3A* gene expression are highest in those tissues that are the primary sites of drug-metabolizing enzymes, namely, liver, intestine, and kidney (Kliewer et al., 1998). Ligands that bind to a given species of PXR correspond to their ability to induce *CYP3A* expression in that species (Jones et al., 2001). PXR binds to specific response elements organized as direct repeats containing the consensus sequence of AGG(T)TCA spaced by three nucleotides (DR3), or to everted repeats spaced by six nucleotides, as a heterodimer with the 9-*cis* retinoic acid receptor (RXR α , NR2B1; Quattrochi et al., 1995; Barwick et al., 1996; Huss et al., 1996; Blumberg et al., 1998). In addition, PXR regulates transcription through binding a direct repeat spaced by four nucleotides (DR4) in the 5'-flanking region of human MDR1, the gene that encodes human P-glycoprotein (Geick et al., 2001; Synold et al., 2001). Recently, Kast et al. (2002) reported that multidrug resistance-associated protein 2 is also a targeted PXR gene. Data generated using a PXR "knockout" mice in this laboratory suggest that PXR is required for induction of mouse *oatp2* by PCN (Staudinger et al., 2001).

Although xenobiotic compounds are routinely cleared by biotransformation, uptake of chemicals by hepatic sinusoidal transporters is critical for some chemicals to enter hepatocytes for subsequent biotransformation and eventual excretion. *Oatp2* is a transporter that functions to mediate the hepatic uptake of a broad array of chemicals and drugs. The molecular mechanism by which PCN induces rat *oatp2* has not been investigated. Therefore, it is hypothesized that induction of *oatp2* by PCN would be due to activation of PXR, which would bind to a PXR response element(s) in the 5'-flanking region of rat *oatp2* gene and activate *oatp2* gene expression. In the present study, a rat *oatp2* genomic clone was isolated, exon-intron junctions of *oatp2* gene were determined, and the 5'-flanking region of *oatp2* was sequenced in an effort to search for a PXR response element(s). The present study demonstrates that induction of *oatp2* by PCN seems to be due to direct interaction of PXR and the 5'-flanking region of rat *oatp2* gene.

Experimental Procedures

Materials. Restriction enzymes, T4 kinase, T4 DNA ligase, Klenow fragment, and terminal transferase were purchased from Invitrogen (Carlsbad, CA), unless otherwise indicated. Zeta probe membrane was obtained from Bio-Rad (Hercules, CA). [α - 32 P]ATP and [γ - 32 P]ATP were obtained from Amersham Biosciences, Inc. (Piscataway, NJ).

Genomic Cloning. A rat genomic bacterial artificial chromosome (BAC) library (BAC Rat Genomic library; Genome Systems, St. Louis, MO) was screened with a 636-bp *ScaI* fragment, containing part of the C-terminal coding sequence and part of the 3'-untranslated region (UTR) from rat *oatp2* cDNA (generously provided by Dr. Peter Meier, University Hospital, Zurich, Switzerland; Genome Systems performed the library hybridization screening). One positive BAC clone containing an approximately 90-kb insert was isolated and confirmed to be a rat *oatp2* genomic clone by partial gene sequencing. This BAC clone was used for sequencing of intron-exon junctions and the 5'-flanking region with an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA), by using primers designed from rat *oatp2* cDNA. Where necessary, sequencing was done with primer walking, by using primers designed from each newly obtained genomic sequence. Oligonucleotide primer synthesis and sequencing reactions were performed at the Biotechnology Support Facility at the University of Kansas Medical Center (Kansas City, KS).

Sequence Analysis. Sequence alignments and analysis were performed with Dnasis software (Dnasis, version 2.1; Hitachi Software Engineering, Yokohama, Japan). The transcription factor-binding sites were predicted with TRANSFAC 4.0 software (<http://transfac.g-bf.de/cgi-bin/matSearch/matsearch2.pl>).

Putative Transcription Start Site Determination. 5'-Rapid amplification of cDNA ends (5'-RACE) was performed to determine the putative transcription start site for rat *oatp2*, by using rat Marathon-Ready cDNA library (CLONTECH, Palo Alto, CA) per the manufacturer's instructions. *Oatp2* gene-specific primer for the RACE was designed and synthesized based on the cDNA sequence of rat *oatp2* cloned by Noe et al. (1997). The 5'-RACE was performed with Adapter primer 1 that came with the kit and *oatp2* gene-specific primer 5'-CCT TCA TAA GAG GTT GTT AAG CCT GCC ACT GGA-3'. PCR was performed using Marathon cDNA amplification kit as follows: 94°C \times 45 s; 5 cycles of 94°C \times 5 s, 72°C \times 4 min; 5 cycles of 94°C \times 5 s, 70°C \times 4 min; 28 cycles of 94°C \times 5 s, 68°C \times 4 min; and a final extension at 72°C \times 10 min. The RACE products were subcloned into pT-Adv vector (CLONTECH) and sequenced with ABI Prism 377 DNA sequencer (Applied Biosystems).

Sequencing of 5'-Flanking Region of Rat *oatp2* Gene. The 5'-flanking region of rat *oatp2* gene was sequenced with primer walking, by using primers designed from each newly obtained genomic sequence. Whenever multiple priming was encountered, the primers were designed from a sequence further to the 3' end of the last sequencing result.

Electrophoretic Mobility Shift Assays. Electrophoretic mobility shift assays were performed as described previously (Goodwin et al., 2001). Rat PXR1 and human RXR α were synthesized in vitro by using the TNT rabbit reticulocyte lysate-coupled in vitro transcription/translation system (Promega, Madison, WI) according to the manufacturer's instructions. Gel mobility shift assays (20 μ l) contained 10 mM Tris, pH 8.0, 40 mM KCl, 0.05% Nonidet P-40, 6% glycerol, 1 mM dithiothreitol, 0.2 μ g of poly(dI-dC), and 2.5 μ l each of in vitro-synthesized PXR and RXR proteins. The total amount of reticulocyte lysate was maintained constant in each reaction (5 μ l) through the addition of unprogrammed lysate. Competitor oligonucleotides were included at 5-, 25-, 50-, or 100-fold excess as indicated in the figure legends. After 10-min incubation on ice, 10 ng of 32 P-labeled oligonucleotide was added and the incubation was continued for an additional 10 min. DNA-protein complexes were resolved on a 4% polyacrylamide gel in 0.5 \times Tris borate-EDTA (1 \times Tris borate-

EDTA is 90 mM Tris, 90 mM boric acid, 2 mM EDTA). Gels were dried and subjected to autoradiography at -80°C . The oligonucleotides used as probes or competitors corresponding to the wild-type and mutant DR3 of CYP3A1, DR3-1, DR3-2, DR3-3, and DR3-4 of oatp2 are detailed in Table 1.

Reporter Gene Construction. Different constructs containing the 5'-flanking region of rat oatp2 were subcloned into pGL3-basic luciferase gene vector (generously provided by Dr. Michael Wolfe, University of Kansas Medical Center), and the sequences of these constructs were confirmed by sequencing.

1. pGL3-basic-oatp2(-8701)-(+49). Oatp2 genomic DNA was digested with restriction enzymes *Sst*I and *Xho*I to produce the -3111 to -8701 segment of the oatp2 promoter, which was confirmed by PCR screening, and was isolated and cloned into PGL-3 basic vector. The -3111 to +49 segment of oatp2 promoter was PCR-amplified (forward primer: 5'-GAT GTT AAT GGT ATG CAC AGG CAG GGA GGC-3'; reverse primer: 5'-AAG AAG CTC GAG AAG ACG GTC CTC TCC ATG C-3'), and subcloned into pT-Adv vector (CLONTECH), which was subsequently digested by *Xho*I, and inserted into the pGL3-basic(-8701)-(-3111), producing the pGL3-basic-oatp2(-8701)-(+49) construct.
2. pGL3-basic-oatp2(-5425)-(+49). pGL3-basic-oatp2(-8701)-(+49) was digested with *Sst*I and *Aat* II, the ends were filled by Klenow enzyme, the larger fragment was gel-purified, and the vector was self-ligated.
3. pGL3-basic-oatp2(-2777)-(+49). pGL3-basic-oatp2(-8701)-(+49) was cut with *Sst*I and *Pst*I, the ends were filled by Klenow enzyme, the larger fragment was gel-purified, and the vector was self-ligated.
4. pGL3-basic-oatp2(-8701)-(-4668). pGL3-basic-oatp2(-8701)-(+49) was cut with *Pst*I and *Nde*I, the ends were filled by Klenow enzyme, the larger fragment was gel-purified, and the vector was self-ligated.
5. pGL3-basic-oatp2(-8701)-(-6978). pGL3-basic-oatp2(-8701)-(-4668) was digested with *Eco*RI, the larger fragment was gel-purified, and the vector was self-ligated.
6. pGL3-basic-oatp2(-5467)-(-4668). pGL3-basic-oatp2(-8701)-(-4668) was digested with *Sst*I and *Eco*R V, the ends were filled by Klenow enzyme, the larger fragment was gel-purified, and the vector was self-ligated.
7. pGL3-basic-oatp2(-8701)-(-5425). pGL3-basic-oatp2(-8701)-(+49) was cut with *Aat* II and *Nde*I, the ends were filled by Klenow enzyme, the larger fragment was gel-purified, and the vector was self-ligated.
8. pGL3-basic-oatp2(-8701)-(-7903)/(-5467)-(-4668). pGL3-basic-oatp2(-8701)-(-4668) was cut with *Pvu*I and *Eco*R V, the ends were filled by Klenow enzyme, the larger fragment was gel-purified, and the vector was self-ligated.
9. pGL3-basic-oatp2(-8203)-(-7903)/(-5467)-(-4668). pGL3-basic-oatp2(-8701)-(-7903)/(-5467)-(-4668) was cut with *Sst*I and *Stu*I, the ends were filled by Klenow enzyme, the larger fragment was gel-purified, and the vector was self-ligated.

Cell Culture and Transient Transfection Assays. Rat hepatoma cell line H4IIE was obtained from American Type Culture Collection (Manassas, VA) and maintained in phenol red-free Dulbecco's modified Eagle's medium (DMEM) (Invitrogen), supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Irvine, CA). The cells were seeded at $1.5 \times 10^5/\text{ml}$ into 12-well plates (500 μl /well) in phenol red-free DMEM, supplemented with 10% charcoal-stripped, delipidated FBS (Sigma Chemical, St. Louis, MO), and 1% penicillin/streptomycin overnight. Cells were transfected by a 4-h exposure to LipofectAMINE^{plus} (Invitrogen), with each well containing 900 ng of the reporter constructs, 500 ng of rPXR expression vector (pSG5-rPXR), and 100 ng of the internal control vector for transfection efficiency, namely, the Rous sarcoma virus promoter- β -galactosidase (pRSV- β -GAL) reporter plasmid (provided by Dr. Michael Wolf, University of Kansas Medical Center) in OptiMEM I (Invitrogen). The media were changed into phenol red-free DMEM, supplemented with 10% charcoal-stripped-delipidated FBS, and 1% penicillin/streptomycin, containing either PCN or vehicle control, DMSO. After a 72-h incubation, the cells were harvested, lysed, and luciferase activity was measured and normalized with β -galactosidase activity. Each reporter construct was assayed in triplicate wells, and each experiment was repeated at least three times.

Statistical Analysis. The data were expressed as mean \pm S.E. Statistical significance between PCN- and vehicle-treated groups was analyzed by Student's *t* test or one-way analysis of variance, followed by Duncan's post hoc analysis. Significance was set at $p < 0.05$.

Results

Genomic Organization of Rat oatp2. There are two existing cDNAs for rat oatp2 (Noe et al., 1997; Abe et al., 1998). The coding regions are the same between these two cDNAs, but their 5'-UTRs are different. To understand the mechanism by which heterogeneous oatp2 mRNAs are produced, as well as to understand regulation of rat oatp2 at the genomic level, a rat BAC library was screened by using a fragment from a rat oatp2 cDNA as a probe. A single BAC clone containing a full span of rat oatp2 gene was isolated and analyzed by partial sequencing. The genomic structure and exon-intron organization relative to rat oatp2 cDNA reported by Abe et al. (1998) are shown in Fig. 1 and Table 2. The rat oatp2 gene contains 16 exons. Exons 1 and 2 contain the first 160 bp of the 5'-UTR of rat oatp2 cDNA cloned by Abe et al. (1998). Exon 3 is 118 bp and contains 58 bp of the 5'-UTR, and the rest of exon 3 encodes the first 20 amino acid residues of rat oatp2 protein. The last exon (exon 16) is the largest, which consists of 1596 bp that contains the stop codon and the 3'-UTR. The initial and terminal dinucleotides of all rat oatp2 introns showed the GT-AG configuration that is characteristic of splice junctions (Lewin, 1997). When the

TABLE 1.
Oligonucleotides for electrophoretic mobility shift assays

Oligonucleotides	Sequence	Length
CYP3A1 DR3	5'GAT GCA GAC AGT TCA TGA AGT TCA TCT AGA TC 3'	32
Mutant CYP3A1 DR3	5'GAT CAG ACA GAA CAT GAA GAA CAT CTA GAT C 3'	31
DR3-1	5'CCA TTT TTA AGC TCA AGA AGT TCA TCA GTA TAG 3'	33
Mutant DR3-1	5'CCA TTT TTA AGA ACA AGA AGA ACA TCA GTA TAG 3'	33
DR3-2	5'GAC ATG GGT GCA CTG AGT CCA CTG ATT AAT T 3'	33
Mutant DR3-2	5'GAC ATG GGT GTT CTG AGT CTT CTG ATT AAT T 3'	33
DR3-3	5'AAT CCC TTT TGA CCT TTA TGC CCT TCA GAA TGC 3'	33
Mutant DR3-3	5'AAT CCC TTT TGT TCT TTA TGT TCT TCA GAA TGC 3'	33
DR3-4	5'GCT GAA GGA GGA TCT GAT TGA ACT AAT ACT GTC 3'	33
Mutant DR3-4	5'GCT GAA GGA GGT TCT GAT TGT TCT AAT ACT GTC 3'	33

sequences of the two published *oatp2* cDNAs were compared with the genomic rat *oatp2* DNA sequence, it was found that the cDNA of rat *oatp2* cloned by Noe et al. (1997) lacked the entire noncoding exon 2, which was included in the cDNA cloned by Abe et al. (1998). Although *oatp2* cDNA by Noe et al. (1997) lacks the entire exon 2, it is longer due to larger contributions from exon 1 (60 versus 30 bp in Abe's cDNA) and exon 16 (1713 versus 814 bp in Abe's cDNA). The phases in the exon-intron boundaries of rat *oatp2* consist of two phase 0, seven phase 1, and four phase 2 splice sites (Table 2).

Analysis of the 5'-Flanking Sequence of Rat *oatp2* Gene. Approximately 8.7 kb of the 5'-flanking region of rat *oatp2* gene were sequenced by primer walking. The sequence of the 5'-flanking region of rat *oatp2* gene is not presented in this article due to its length, but is available in the GenBank as accession number AF426312. The putative transcription start site was estimated by the 5'-RACE assay, by using rat *oatp2* gene-specific primer designed from the 5'-UTR region, and tentatively determined by the longest RACE product. The estimated transcription start site is 89 bp from the start of intron 1, and is 29 bp upstream of the reported rat *oatp2* cDNA cloned by Noe et al. (1997). Therefore, the calculated

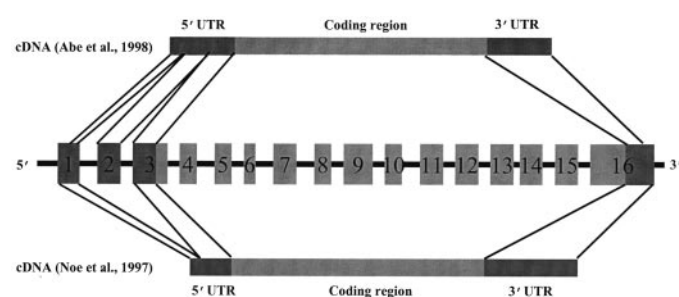


Fig. 1. Exon-intron organization of the rat *oatp2* gene and its organizational relationship to the two published *oatp2* cDNAs cloned by Noe et al. (1997) and Abe et al. (1998), respectively. Exons are arranged from the 5' to the 3' end. Dark gray boxes represent noncoding exons and light gray boxes represent coding exons. Black lines between exons represent introns (without determination of their length). The cDNA cloned by Noe et al. (1997) lacks the entire exon 2, which is present in the cDNA cloned by Abe et al. (1998). The first and last exon contribute longer sequence to the cDNA cloned by Noe et al. (1997).

TABLE 2.

Structure of Rat *Oatp2* Gene

The length of the first exon is 89 bp, from which 30 bp is in the cDNA cloned by Abe et al. (1998), and 60 bp in the cDNA cloned by Noe et al. (1997).

Exon	Size in cDNA bp	Position in cDNA	Phase
1	30	1–30	N.D.
2	130	31–160	N.D.
3	118	161–278	0
4	141	279–420	1
5	132	421–553	2
6	104	554–658	1
7	146	659–805	1
8	98	806–904	1
9	221	905–1126	1
10	164	1127–1291	1
11	195	1292–1487	2
12	165	1488–1653	0
13	172	1654–1826	2
14	64	1827–1891	1
15	117	1892–2009	2
16	814	2010–2824	N.D.

N.D., not determined.

5'-UTR would be 277 bp if all noncoding exons (exons 1, 2, and 3) are present. A TATA box for eukaryotic promoters is absent in the proximal 5'-flanking region of the rat *oatp2* gene. Multiple repeats of alternative purine pyrimidine (AC or AG) were present in the proximal promoter of rat *oatp2* (–263 to –421), which is also present in the mouse *oatp2* promoter region (Ogura et al., 2001). Potential binding sites for many transcription factors were identified in the 5'-flanking region of rat *oatp2* gene detected by TRANSFAC, including three matches to aryl hydrocarbon receptor/aryl hydrocarbon receptor nuclear translocator (AhR-Arnt) protein, three matches to chicken ovalbumin upstream promoter transcription factor, four matches to cyclic AMP response element binding protein, one match to glucocorticoid response element, four matches to hepatocyte nuclear factor (HNF) 1, 85 matches to HNF3B, seven matches to NF-κB, one match to peroxisome proliferator-activated receptor α, and 12 matches to signal transducers and activators of transcription (STAT) 1, etc. The presence of the above-mentioned *trans*-activator binding sites suggests their functional involvement in the transcriptional control of the rat *oatp2* gene. However, further experiments are required to determine the regulation of rat *oatp2* by these transcription factors at the functional level.

Four potential PXR response elements, arranged as direct repeats separated by three nucleotides (DR3) were identified in the 5'-flanking region of rat *oatp2* gene; one was identified at –5000 bp (DR3-1), and the other three clustered around the –8000-bp region (DR3-2, DR3-3, and DR3-4) (Fig. 2). In comparison with the DR3 [AGG(T)TCAnnnAGG(T)TCA] identified in the 5'-flanking region of rat *CYP3A1* (Huss and Kasper, 1998), rat *oatp2* DR3s differ from it by one to three mismatches, with one mismatch in DR3-1 and DR3-3, two mismatches in DR3-4, and three mismatches in DR3-2 (Table 3).

Characterization of Binding Activity of PXR-RXRα Heterodimers to PXR Response Elements Found in 5'-Flanking Region of Rat *oatp2*. PXR binds as a PXR-RXRα heterodimer to AGG(T)TCA hexamers arranged as direct repeats (DR3 or DR4) or everted repeat 6 identified in the 5'-flanking region of *CYP3A* and *MDR1* genes (Bertilsson

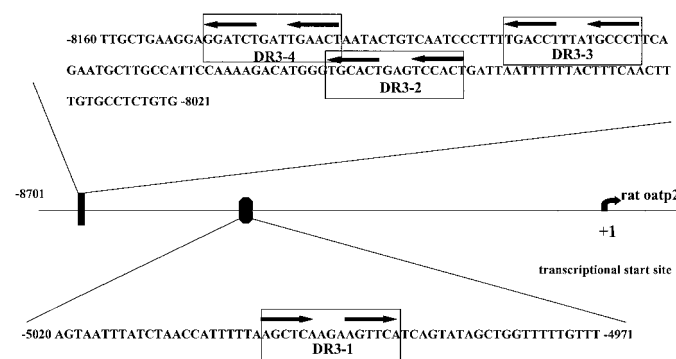


Fig. 2. 5'-Flanking region of rat *oatp2* gene and position of putative PXR response elements (DR3s). The figure shows the position of the putative PXR response elements (DR3s) in the 5'-flanking region of the rat *oatp2* gene in relation to the transcription start site (+1). Four PXR response elements are numbered DR3-1, DR3-2, DR3-3, and DR3-4, according to the sequence by which they were identified. DR3-1 was identified at 5 kb upstream of the transcription start site (+1), whereas DR3-2, DR3-3, and DR3-4 were identified as a cluster around 8 kb upstream of the transcription start site (+1).

et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998). Because PXR seems to have a promiscuous ability to bind multiple hormone response elements, the ability of PXR to bind the four identified potential PXREs in the 5'-flanking region of the rat *oatp2* gene was assessed. Binding of the radiolabeled rat CYP3A1 PXR response element (PXRE) was abolished by incubation with excess nonradiolabeled rat CYP3A1 DR3 (Fig. 3). Competition with 5-, 25-, and 100-fold molar excess of the four putative PXREs (DR3-1 through DR3-4) revealed that DR3-2 binds the most efficiently, followed by DR3-4 and DR3-1. DR3-3 bound very weakly or not at all. The mutant form (Δ DR3s) of all of the PXREs did not compete with the binding of the PXR-RXR α heterodimer to CYP3A1 DR3.

Because the PXR-RXR heterodimer bound most efficiently to the DR3-2, this element was labeled with 32 P in an effort to directly assay binding of the PXR-RXR heterodimer to this element. PXR-RXR α heterodimer bound to DR3-2 and the binding was abolished by the addition of 5- and 50-fold molar excess of nonradiolabeled rat CYP3A1 oligonucleotide (Fig. 4). Competition experiments with unlabeled DR3-1 through DR3-4 oligonucleotides in a 5- and 50-fold molar excess revealed that DR3-2 competes the most efficiently, followed by DR3-4 and DR3-1, whereas the DR3-3 oligonucleotide competed only weakly if at all. In addition, the binding was not competed by the presence of excess unlabeled oligonucleotides containing mutant forms of DR3s from CYP3A1 (Δ CYP3A1) and *oatp2* DR3s (Δ DR3-1, Δ DR3-2, Δ DR3-3, and Δ DR3-4). Direct binding study of the PXR-RXR α heterodimer with the 32 P-labeled DR3-1, DR3-3, and DR3-4 was also performed, and the results confirmed what were observed in the aforementioned experiments (data not shown).

PXR Trans-Activates Rat *oatp2* Promoter. PXR has been shown to *trans*-activate the CYP3A1 promoter by using PCN as a model chemical. To determine whether PXR mediates the *trans*-activation of the *oatp2* promoter in the presence of PCN, an 8.7-kb 5'-flanking region of *oatp2* (with DR3-1, DR3-2, DR3-3, and DR3-4) was cloned into a PGL3-basic vector, upstream of the luciferase reporter gene. The construct was transiently transfected into the rat hepatoma cell line H4IIE with concomitant cotransfection of pSG5-rat PXR, and in the presence of PCN at 0.1, 1, 10, and 100 μ M concentrations or vehicle (0.1% DMSO). As shown in Fig. 5, PCN dose dependently activated the 8.7-kb 5'-flanking region of the rat *oatp2* gene, and approximately 11-fold induction was observed with 100 μ M PCN.

Minimum of 300 bp around -8-kb Region of 5'-Flanking Sequence of Rat *oatp2* Is Required for PCN Re-

sponse. To determine which region(s) in the 5'-flanking region of *oatp2* is responsible for *trans*-activation by PCN, series deletions to generate different lengths of the 5'-flanking region of rat *oatp2* gene (constructs 1-9) were made (Fig. 6). They were transiently transfected into H4IIE cells with pSG5-rat PXR in the presence of 100 μ M PCN. The deletion of the upstream region of -5425 (-5425 to -8700) significantly decreased the induction by PCN from 8- to 2.5-fold (construct 2, with DR3-1). Partial deletion of the downstream region of -5425 (-2777 to -5425) did not affect the degree of induction (2.5- to 2.5-fold) (construct 3, no DR3s). Deletion of the proximal portion of the 5'-flanking region of *oatp2* (-369 to -4668) seems to increase the degree of induction (8- to 13.4-fold), suggesting the presence of a suppressive region between -369 to -4668 (construct 4, with DR3-1, DR3-2, DR3-3, and DR3-4). Deletion of -4696 to -6978 from the

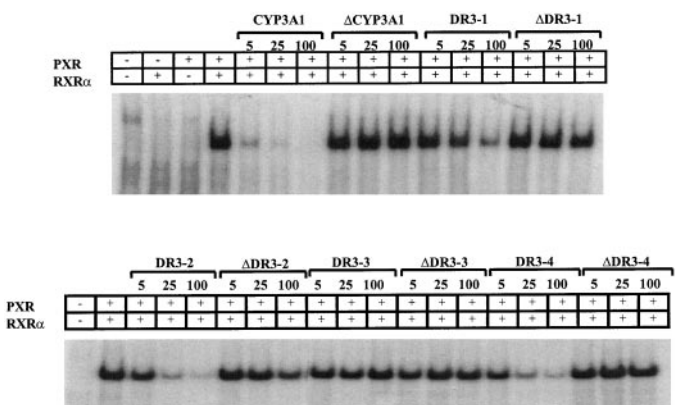


Fig. 3. DR3s from rat *oatp2* gene compete with the binding of the PXR/RXR α heterodimer to the CYP3A1-DR3. Electrophoretic mobility shift assays using in vitro-translated proteins bound to 32 P-labeled oligonucleotides containing the DR3 from the 5'-flanking region of rat CYP3A1. Binding reactions contained (+) or lacked (-) the indicated proteins. The figure illustrates the PXR, RXR α , or PXR-RXR α heterodimer binding to 32 P-labeled DR3 from CYP3A1, as well as the competition with wild-type and mutant (Δ) unlabeled oligonucleotides containing the DR3 from the 5'-flanking region of CYP3A1, or DR3-1, DR3-2, DR3-3, and DR3-4 from the 5'-flanking region of rat *oatp2*. The numbers indicate the *n*-fold molar excess to which the competitor was added.

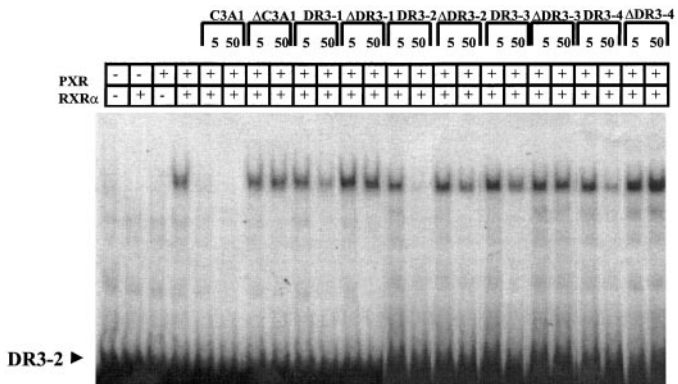


Fig. 4. Characterization of the binding of the PXR/RXR α heterodimer to the *oatp2* DR3-2. Electrophoretic mobility shift assays using in vitro-translated proteins bound to 32 P-labeled oligonucleotides containing the DR3-2 from the 5'-flanking region of the rat *oatp2* gene. Binding reactions contained (+) or lacked (-) the indicated proteins. The figure illustrates the PXR, RXR α , or PXR-RXR α heterodimer binding to 32 P-labeled DR3-2, as well as the competition with wild-type and mutant (Δ) unlabeled oligonucleotides containing the CYP3A1 DR3, rat *oatp2* DR3-1, DR3-2, DR3-3, and DR3-4. The numbers indicate the *n*-fold molar excess to which the competitor was added.

TABLE 3.

Comparison of the DR3s from the rat CYP3A1 and *oatp2*

	Sequence	Mismatch
Perfect DR3 (sense)	5' AGT(T)TCAnnnAGT(T)TCA 3'	
Perfect DR3 (antisense)	5' TGA(A)ACTnnnTGA(A)ACT 3'	
CYP3A1 DR3 (sense)	5' AGTTCAtgaAGTTCA 3'	
CYP3A1 DR3 (antisense)	5' TGAACCTtcaTGAAC 3'	
DR3-1 (sense)	5' AGCTCAagaAGTTCA 3'	1
DR3-1 (antisense)	5' TGAACCTtctTGAGCT 3'	
DR3-2 (sense)	5' TGCACTgagTCCACT 3'	3
DR3-2 (antisense)	5' AGTGGActcAGTGCA 3'	
DR3-3 (sense)	5' TGACCTttaTGCCCT 3'	1
DR3-3 (antisense)	5' AGGGCataaAGGTCA 3'	
DR3-4 (sense)	5' GGATCTgatTGAAC 3'	2
DR3-4 (antisense)	5' AGTTCAatcAGATCC 3'	

backbone of construct 4, resulting in lack of the DR3-1, reduced the fold induction from 13.4- to 10.7-fold (construct 5, with DR3-2, DR3-3, and DR3-4). Deletion of -5467 to -8700 from the backbone of construct 4 resulted in loss of the three distal DR3s (DR3-2, DR3-3, and DR3-4), and the fold induction significantly reduced from 13.4- to 2.4-fold (construct 6, with DR3-1). Inclusion of the region between -5425 to -8700 (construct 7) increased induction (2.4- to 5.3-fold); however, the degree was still lower than construct 5, indicating the presence of a suppressive region between DR3-1 and the three other DR3s. Deletion of the region between -5467 and -7903 (construct 8, with DR3-1, DR3-2, DR3-3, and DR3-4)

restored the induction by PCN (13.2-fold). Further deletion from the 5' end of construct 8 (-8203 to -8700) yielded a somewhat stronger induction (18.8-fold) (construct 9), suggesting the existence of another suppressive region between -8203 to -8700. These data demonstrate that the distal region in the 5'-flanking region of *oatp2* gene, which contains the three putative DR3s (DR3-2, DR3-3, and DR3-4), was more responsive to the induction of PCN than the relatively proximal region where DR3-1 was identified.

Discussion

Rat *oatp2*, a hepatic sinusoidal transporter, mediates hepatic uptake of a variety of structurally diverse compounds. Although its substrate spectrum overlaps with other *oatp* family members, its affinity for cardiac glycosides, such as ouabain and digoxin, is very high (Noe et al., 1997; Abe et al., 1998; Kakyo et al., 1999). Expression levels of rat hepatic *oatp2* protein and mRNA are increased in adult and newborn rats by treatment with PCN (Rausch-Derra et al., 2001). Moreover, increased *oatp2* gene transcription seems to be responsible for the increased *oatp2* mRNA expression after PCN treatment (Guo et al., 2001). PXR is the PCN receptor and its activation correlates with the induction of CYP3A family members in all species tested (Jones et al., 2001), and PCN induction of mouse *oatp2* mRNA was abolished in PXR knockout mice (Staudinger et al., 2001). Therefore, it was hypothesized that PCN induction of rat *oatp2* would be through interaction with PXR as well.

In the current study, a rat *oatp2* genomic clone was isolated, and analysis of the genomic clone indicated that the rat *oatp2* gene consists of 16 exons (Table 2). The intron and exon boundaries all followed the GT-AG rule for intron splicing. Comparison of the two published cDNAs for rat *oatp2* showed

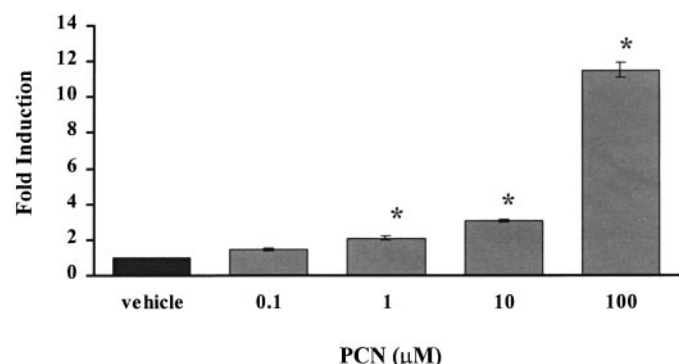


Fig. 5. Trans-activation of rat *oatp2* promoter by PXR in the presence of PCN. Approximately 8.7 kb of the 5'-flanking region of rat *oatp2* were cloned into PGL3-basic vector. The construct was transiently transfected into the rat hepatoma cell line H4IIE with rat PXR and RSV- β -gal vector. The cells were incubated with PCN at 0.1, 1, 10, or 100 μ M or vehicle (DMSO) for 72 h. Cells were harvested and analyzed for luciferase and β -galactosidase activity. The data are presented as fold induction (PCN[luciferase activity/ β -galactosidase activity])/[DMSO(luciferase activity/ β -galactosidase activity)]. Asterisks (*) denote statistical significance between vehicle and PCN-treated groups ($p < 0.05$). The experiments were done in triplicate wells and repeated three times.

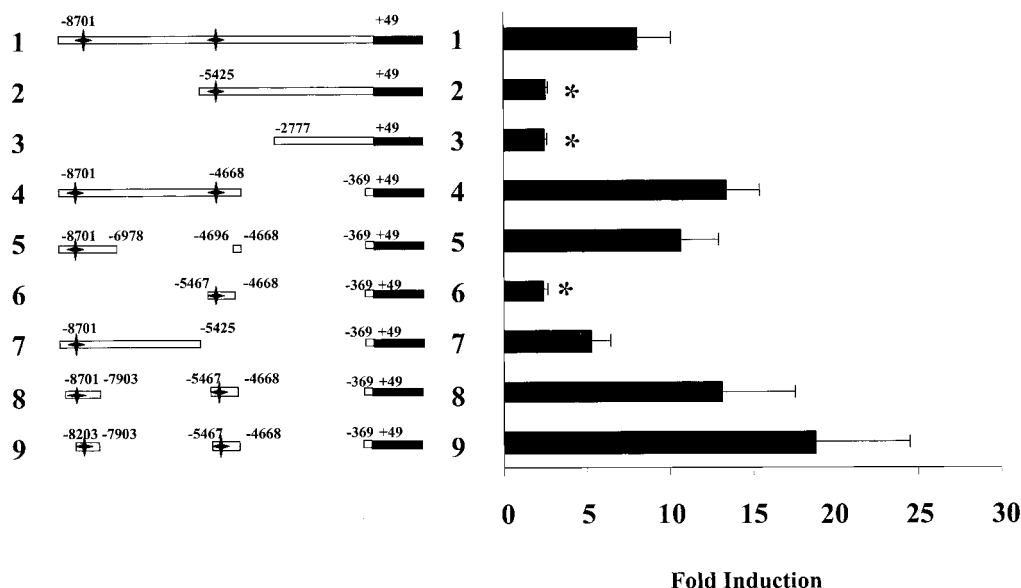


Fig. 6. Trans-activation of the 8.7-kb 5'-flanking region of rat *oatp2* by PXR in the presence of PCN requires a 300-bp region around -8 kb (-8203 to -7903). Series deletions within the 5'-flanking region of rat *oatp2* were made by restriction enzyme digestion, followed by self-ligation of the vector. The sequences were confirmed by sequencing. Structures of different constructs are illustrated on the left side of the graph, numbered from 1 to 9. These constructs were transiently transfected into the H4IIE cells, with rat PXR and RSV- β -gal vector. The cells were incubated with PCN at 100 μ M for 72 h. Cells were harvested and analyzed for luciferase and β -galactosidase activity. The data are presented as fold induction by PCN [PCN(luciferase activity/ β -galactosidase activity))/[DMSO(luciferase activity/ β -galactosidase activity)]. Asterisks (*) denote statistical significance between intact and deleted constructs of the 5'-flanking region of rat *oatp2* ($p < 0.05$). The experiments were done in triplicate wells and repeated three to four times.

that although the coding region of these two oatp2 cDNAs is the same, their 5'-UTRs are different (Noe et al., 1997; Abe et al., 1998). The analysis of rat oatp2 gene structure clearly shows that the cDNA cloned by Noe et al. (1997) lacked the second noncoding exon (exon 2) that was present in the cDNA cloned by Abe et al. (1998). Therefore, the two cDNAs are the result of alternative splicing of the noncoding exon of the rat oatp2 gene (Fig. 1). Research from this laboratory previously indicated the presence of heterogeneous 5'-UTR of the mouse oatp2 gene, due to alternative splicing (Ogura et al., 2001).

Exon-intron structure of rat oatp2 is similar to other members of the oatp family, namely, mouse oatp2 (Ogura et al., 2001); mouse lst-1 (Ogura et al., 2000); rat lst-1 (Choudhuri et al., 2000); human OATP-A, OATP-C, and OATP8 (Konig et al., 2000); and PGT genes (Lu and Schuster, 1998). Gene structure analysis indicates that the coding sequences of these genes are all separated by 13 introns. Mouse oatp2 gene consists of 17 exons, which is one exon longer than rat oatp2, due to the presence of an additional noncoding exon in the mouse oatp2 gene (Ogura et al., 2001). The phases in exon-intron junction are identical among the aforementioned oatp family members, all consisting of two phase 0, seven phase 1, and four phase 2 splice sites. This indicates that these genes may have derived from a common ancestral gene that was converted into different genes during evolution. Because there is a cluster of phase 1 from exon 6 through exon 10, there is the possibility for alternatively spliced forms of rat oatp2 mRNA.

Approximately 8.7 kb of the 5'-flanking region of the rat oatp2 gene were sequenced by primer walking after the putative transcription start site was identified by the 5'-RACE assay. Sequence analysis revealed many putative transcription factor-binding sites, including AhR-Arnt, HNF1, HNF3B, NF- κ B, peroxisome proliferator-activated receptor α , and STAT1. The numerous HNF1 and HNF3B binding sites (4 and 85 matches, respectively) in the 5'-flanking region of the rat oatp2 gene might explain the high level expression of oatp2 in liver. In addition, the observation that rat hepatic oatp2 protein expression is down-regulated by AhR ligands (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, indole-3-carbinol, β -naphthoflavone, and polychlorinated biphenyl 126) (Guo et al., 2002) suggests that this might be due to the presence of AhR-Arnt in the promoter region of the rat oatp2 gene. Moreover, the presence of NF- κ B and STAT in the rat oatp2 promoter suggests that rat oatp2 might be subject to their regulation. These putative transcription factor-binding sites need to be further characterized by functional assays to determine potential mechanisms by which rat oatp2 is regulated by xenobiotics, pathophysiological conditions, and tissue specificity at the molecular level.

Rat hepatic oatp2 protein and mRNA levels are induced by PCN treatment in both adult and newborn animals (Klaassen et al., 2001; Raush-Derra et al., 2001). PCN treatment induces hepatoprotection in rodents (Selye, 1971), and UDP-glucuronosyltransferase in rats (Watkins and Klaassen, 1982). Analysis of 8.7 kb of the 5'-flanking region revealed four putative DR3s in the promoter of the rat oatp2 gene. One DR3 (DR3-1) is located at around -5000 bp in the upstream region, whereas the other three (DR3-2, DR3-3, and DR3-4) are clustered together at about -8000 bp upstream of the putative transcription start site. Electrophoretic mobility shift assay analysis revealed that the PXR-RXR heterodimer

binds the most efficiently to DR3-2, whereas it binds weakly to DR3-1 and DR3-4, or not at all to DR3-3.

Transient transfection studies using rat PXR and the rat oatp2 promoter linked to the luciferase reporter gene reveal that this promoter is induced by PCN treatment in a dose-dependent manner that is dependent upon the presence of rat PXR. A series of deletion mutations established that both the proximal PXRE (DR3-1) and the distal PXREs (DR3-2 through DR3-4) are required for maximal induction of this promoter by PCN. A minimum of 300 base pairs that includes the entire distal cluster of putative PXREs is sufficient and required for induction by PCN.

In summary, this study indicates that the mechanism by which PCN induces rat oatp2 gene is via interaction with PXR. This research furthers our understanding of the coordinate regulation of a biochemically linked set of genes that is important for xenobiotic disposition and elimination, including oatp2, CYP3A, MDR1, and multidrug resistance-associated protein 2 whose expression is induced in a coordinate manner so as to reduce the intracellular concentration of drugs, steroids, and bile acids. In addition, the results aid in understanding more about drug-drug interactions at the hepatic uptake level.

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