Unique Transcription Start Sites and Distinct Promoter Regions Differentiate the Pregnane X Receptor (PXR) Isoforms PXR 1 and PXR 2

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ABSTRACT:
The pregnane X receptor (PXR) is known as the xenosensing receptor responsible for coordinated regulation of metabolic genes in response to diverse xenobiotic challenges. In particular, the ability of the PXR to regulate CYP3A4, the enzyme capable of metabolizing more than 60% of all pharmaceuticals, defines its metabolic importance. Currently the list of PXR ligands and target genes is extensive, yet investigations into the regulation and expression of PXRs are few. After an initial review of available sequence data, we discovered discrepancies in the 5′ untranslated region (UTR) and transcriptional start site (TSS) characterizations of the human PXR gene and subsequently endeavored to define TSSs and proximal promoters for isoforms PXR 1 and PXR 2. Reverse transcriptase-polymerase chain reaction and primer extension experiments performed on RNA from human liver identified two TSSs for each receptor isoform. These results extended the 5′ UTR sequence of each isoform and defined new proximal promoters for both. Candidate response elements for liver-enriched transcription factors and other receptors were found in both proximal promoters. Quantitative PCR from human liver illustrated a highly variable expression profile for total PXRs; yet PXR 2 expression represented a consistent 2 to 5% of total PXR expression, despite the observed variability. Transfection experiments demonstrated that PXR 1 and PXR 2 had comparable abilities to transcriptionally activate the CYP3A4 promoter. Collectively, comparable function, consistent expression, and independent regulation suggest that PXR 2 is capable of contributing to the cumulative function of PXRs and should be included in the larger investigations of PXR expression and regulation.

The data presented here form part of the dissertation of Leslie M. Tompkins that was submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

ABBREVIATIONS: PXR, pregnane X receptor; P450, cytochrome P450; GR, glucocorticoid receptor; PPAR, peroxisome proliferator activated receptor; TSS, transcription start site; RT, reverse transcription; PCR, polymerase chain reaction; kb, kilobase(s); nt, nucleotide(s); RU486, 17β-hydroxy-11β-[4-dimethylamino phenyl]-17α-[1-propynylestra-4,9-dien-3-one; PBDE-71, polybrominated diphenyl ether-71; ANOVA, analysis of variance; NCBI, National Center for Biotechnology Information; bp, base pair(s); PR, progesterone receptor; 5′UTR, 5′ untranslated region.
found to be capable of binding the wide variety of CYP3A inducers. Notorious among the CYP3A xenobiotic inducers was the synthetic glucocorticoid dexamethasone whose signaling was not mediated directly by the glucocorticoid receptor (GR, NR3C1). Pascucci et al. (2001) suggested that the direct effect of the GR was to up-regulate PXR expression, which led to significant induction of CYP3A4 expression. The ability of other nuclear receptors to alter PXR expression adds additional complexity to the regulation of this critical metabolic pathway. Other nuclear receptors [farnesoid X receptor (NR1H4) (Jung et al., 2006), peroxisome proliferator activated receptor (PPAR, NR1C) (Aouabdi et al., 2006), and hepatocyte nuclear factor 4 (NR2A1) (Li et al., 2000)] may participate in the regulation of PXR expression.

In accordance with its participation in regulating metabolism, abundant expression of PXR was initially characterized in the liver and small intestine (Kliewer et al., 1998). To date, PXR mRNA expression has also been reported in the stomach, heart, brain, and breast tissue (Dotzlaw et al., 1999; Lamba et al., 2004). In each of these tissues, PXR levels, the variety of expressed isoforms, and the regulation of that expression have not been fully characterized. Transcript isoforms generated from alternative mRNA processing (splicing, exon usage, and alternate promoters) allow for greater protein diversity and targeted tissue-specific expression from a single gene. These alternate mRNA processing mechanisms are common to the nuclear receptor superfamily as receptors including GR, PPAR, and PXR are expressed as a number of transcript isoforms. PXR research has focused on the 434-amino acid isoform 1 (PXR 1) initially identified by Lehmann et al. (1998), Bertilsson et al. (1998), and Blumberg et al. (1998). However, Bertilsson et al. (1998) reported the discovery of an additional isoform with a distinct 5’ region that resulted in a 473-amino acid isoform known as PAR.2 or PXR 2. Isoforms PXR 1 and PXR 2 share exons 2 through 9 in common, assuring that their ligand- and DNA-binding characteristics are similar, but distinct first exons are responsible for their differences. Multiple transcripts of a single gene that differ in their 5’ region suggest the presence of unique proximal promoters that independently regulate each transcript (Landry et al., 2003).

Based on these observations, the objectives of this research were to fully characterize the unique 5’ regions of each PXR 1 and PXR 2 transcript and identify their distinct promoter regions. The majority of available sequence information was produced via high-throughput methodologies and cDNA library screenings. We sought to characterize the PXR transcripts directly from human liver tissue. In this study, we were able to identify novel transcription start sites (TSSs) for PXR 1 and PXR 2 by classic molecular methods and characterize each sample by its PXR isoform profile. Additionally, reporter assays using a CYP3A4 promoter construct demonstrated that PXR 2 has transcriptional activity comparable to that of PXR 1. In total, these data illustrate a need for better understanding of PXR regulation, isoform expression, and the cumulative effect on the regulation of xenobiotic metabolism.

### Materials and Methods

**Human RNA Samples.** Whole human liver tissue samples (5–500 g) were purchased from National Disease Research Interchange (Philadelphia, PA). Tissue was snap-frozen in liquid N₂ within 6 to 8 h of expiration, according to our submitted protocol. Total RNA was harvested using the RNeasy Maxi kit (QIAGEN, Valencia, CA).

**5’ RT-PCR of PXR 1 and PXR 2.** Total RNA harvested from human liver samples was reverse-transcribed using the ImProm II RT System (Promega, Madison, WI). PCR was done using the Hi-Fidelity PCR PLUS system (Roche Diagnostics, Indianapolis, IN), and cycling parameters were optimized for each primer pair. PCR primers (IDT, Coralville, IA) are shown in Table 1. All PCR products were TA-cloned (Invitrogen, Carlsbad, CA) and sequenced (SeqWright, Houston, TX). BLASTn (http://www.ncbi.nlm.nih.gov) was used to ensure identity with PXR sequences: AF364606 (genomic), NM_003889 (PXR 1 cDNA), and NM_022002 (PXR 2 cDNA).

**Primer Extension.** Primer extension reactions were designed to confirm PXR-determined TSSs with two oligos (20- to 23-mer) for each transcript. For quantitative comparison, 10 pmol of each primer (Table 1) was end-labeling with [γ-32P]ATP (specific activity 3000 Ci/mmol) using T4 polynucleotide kinase according to the manufacturer’s instructions (Promega). After heating with 0.5 M EDTA, 20 μg of total RNA from human liver was added, and the sample was precipitated with 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol at −20°C for 30 min to overnight. To anneal, the RNA and primer were resuspended in dH₂O and incubated at 70°C for 10 min followed by snap-cooling on ice. The extension reaction was performed with Affinity-Script Reverse Transcriptase (Stratagene, La Jolla, CA) under the manufacturer’s suggested conditions with the following modifications: 2.0 mM dNTP mix (0.5 mM concentrations of each dNTP) and 15 U of RNasin (ribonuclease inhibitor; Promega). The reaction was incubated at 42°C for 30 min and quenched with 1 volume of 2X loading dye (95% formamide, 18 mM EDTA, 0.025% SDS, xylene cyanol, and bromphenol blue). Each sample was heated to 90°C for 10 min before loading on the sequencing gel.

A 2.5 kb genomic region (containing exon 1 of both PXR 1 and PXR 2) was TA-cloned (Invitrogen) and used as template for the sequencing ladder. The 2.5 kb genomic construct was sequenced (SeqWright) to identify the sequence of each PXR promoter that contained its own promoters.

### Table 1: All oligonucleotide primers

<table>
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<tr>
<th>Name</th>
<th>Sequence (5’ → 3’)</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>PXR1.1</td>
<td>AGGACCTAGAAGAAGACGCA</td>
<td>45895</td>
</tr>
<tr>
<td>PXR1.2</td>
<td>GCTAGAAGACGACAAGTCCCA</td>
<td>45598</td>
</tr>
<tr>
<td>PXR1.3</td>
<td>CAGCTTACGGGATGTTCCCTTC</td>
<td>45524</td>
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<tr>
<td>PXR1.4</td>
<td>CACCTACAAGTTCCCCTCCCT</td>
<td>45415</td>
</tr>
<tr>
<td>PXR2.1</td>
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<td>46524</td>
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<tr>
<td>PXR2.2</td>
<td>CTTCTAGATCTACTTGAAGAAA</td>
<td>46338</td>
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<td>PXR2.3</td>
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<td>PXR2.4</td>
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<td>70510</td>
</tr>
<tr>
<td>PXR1A</td>
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<td>45686</td>
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<tr>
<td>PXR1B</td>
<td>TGCCCCCTTGGCTGACAGAG</td>
<td>46015</td>
</tr>
<tr>
<td>PXR2A</td>
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</tr>
<tr>
<td>PXR2B</td>
<td>CTGGGGGTTGATAGAGTTGCTG</td>
<td>46470</td>
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</table>

The CYP3A4 promoter (46470–32P]ATP (specific activity 3000 Ci/mmol) using T4 polynucleotide kinase according to the manufacturer’s instructions (Promega). After heating with 0.5 M EDTA, 20 μg of total RNA from human liver was added, and the sample was precipitated with 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol at −20°C for 30 min to overnight. To anneal, the RNA and primer were resuspended in dH₂O and incubated at 70°C for 10 min followed by snap-cooling on ice. The extension reaction was performed with Affinity-Script Reverse Transcriptase (Stratagene, La Jolla, CA) under the manufacturer’s suggested conditions with the following modifications: 2.0 mM dNTP mix (0.5 mM concentrations of each dNTP) and 15 U of RNasin (ribonuclease inhibitor; Promega). The reaction was incubated at 42°C for 30 min and quenched with 1 volume of 2X loading dye (95% formamide, 18 mM EDTA, 0.025% SDS, xylene cyanol, and bromphenol blue). Each sample was heated to 90°C for 10 min before loading on the sequencing gel.

A 2.5 kb genomic region (containing exon 1 of both PXR 1 and PXR 2) was TA-cloned (Invitrogen) and used as template for the sequencing ladder. The 2.5 kb genomic construct was sequenced (SeqWright) to identify the sequence of each PXR promoter that contained its own promoters.

**Quantitation of PXR Isoforms by Real-Time PCR.** Total RNA from human liver was isolated and reverse-transcribed as above. TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) for PXR (PXR 2-specific (Hs00243654_m1) and total PXR (Hs00243666_m1) and ribosomal 18S subunit were performed according to the manufacturer’s instructions.

**Transient Transfection Assays.** HepG2 cells (human hepatoblastoma cells; American Type Culture Collection, Manassas, VA) were plated at 3.0 × 10⁵ cells/well. Twenty-four hours later, cells were transfected with 1.0 μg of CYP3A4-luc and 0.1 μg of empty vector pcDNA3.1(+) or pcPXR1 or pcPXR2 using TransIT-LT1 reagent (Mirus Bio Corp., Madison, WI) in 10⁵ cells/well. Twenty-four hours after transfection, cells were lysed and luciferase activity was quantitated with a luminometer (Tecan).
Opti-MEM I reduced serum medium (Invitrogen). Four to 6 h later, medium was removed, and cells were allowed to recover overnight in cellgro COMPLETE medium. Cells were treated for 48 h with vehicle control, dimethyl sulfoxide, and 10 μM rifampicin, 10 μM lithocholic acid (Sigma-Aldrich, St. Louis, MO), 10 nM dexamethasone, 10 μM RU486, 10 μM cortisol (Steroids Newport, RI), or 10 μM polybrominated diphenyl ether-71 (PBDE-71) (a kind gift of Drs. Thomas Burka and J. Michael Sanders, National Institute of Environmental Health Sciences, Research Triangle Park, NC). After treatment, cells were lysed and harvested in 1× Reporter Lysis buffer and processed according to the Luciferase Assay System (Promega). Lysates were measured on a TD-20/20 Luminometer (Turner Designs, Inc., Sunnyvale, CA) and normalized to total protein concentration. Luciferase data are expressed as fold induction over vehicle-treated control. All statistical analysis was performed using JMP software (version 6.0.0; SAS Institute, Inc., Cary, NC). Comparisons between treatment and control groups were made using ANOVA followed by Dunnett’s multiple comparison test with a significance level of 0.05. Comparisons of CYP3A4 reporter activity between treatments of PXR 1 and PXR 2 transfected cells were made using ANOVA followed by the Tukey-Kramer honestly significant difference with a significance level of 0.05. Comparisons between treatment and control groups were made using ANOVA followed by Dunnett’s multiple comparison test with a significance level of 0.05. 

**Results**

**RT-PCR to Map 5’ Regions of PXR 1 and PXR 2.** We began our investigation of the 5’ regions of PXR 1 and PXR 2 with analysis of sequences available from the NCBI database. However, most sequences shared little to no consensus concerning the size of the 5’ region or a TSS. It remained unclear whether a start of transcription for any sequence had been experimentally determined. Using the genomic *pregnane X receptor* sequence, AF364606 (Zhang et al., 2001) with 45 kb of promoter, we were able to design primers for RT-PCR as opposed to using a 5’ rapid amplification of cDNA ends technique to explore an undetermined 5’ region. Our forward primers were designed to span the known and unknown regions at the 5’ end of PXR 1 and PXR 2 message with the reverse primer nested in the common exon 2 to control for genomic contamination. PCR products of predicted size would be observed unless the sequence of the next 5’ primer was not contained in the transcript, thus identifying a putative region for a TSS (Fig. 1). Forward primers PXR1.1 through PXR1.4 were used to characterize PXR 1 (Fig. 1A), and products of the expected size were seen with primers PXR1.1, 1.2, and 1.3. Primer 1.4 failed to yield the expected sized PCR product, indicating a putative TSS region. Likewise, forward primers PXR2.1 through PXR2.4 were used to characterize PXR 2 (Fig. 1B), and products of expected size were seen with primers PXR2.1, 2.2, and 2.3. Primer PXR2.4 failed to yield the expected sized product but did generate a much smaller amplification product. As illustrated in the map in Fig. 1, primer PXR2.4 annealed in the terminal 25 nt of PXR 1, exon 1, and this small PCR product was sequenced and discovered to be a product of the PXR 1 transcript where PXR 1, exon 1 is spliced to exon 2. With respect to the mapping of PXR 2, the result of primer PXR2.4 confirms that the primer sequence is not part of the PXR 2 transcript and brackets the region between primer PXR2.3 as a likely site for a start of transcription. Our strategic design of PCR primers identified a ~110-bp region and a ~160-bp region for PXR 1 and PXR 2, respectively, where a TSS should be present.

**Primer Extension of PXR 1 and PXR 2 Transcripts.** On the basis of conclusions drawn from the RT-PCR data, new reverse primers were designed to determine TSSs by primer extension analysis (Fig. 2). The 5’ region of PXR 1 was investigated using the primers PXR1A and PXR1B, and similarly PXR 2 was investigated by primers PXR2A and PXR2B (Table 1). The PXR1A primer extension reaction generated a single extension product, which appears as a doublet corresponding to GA residues in the ladder. The doublet represents a stutter at the 5’ G-cap suggesting a fully capped and mature message, supporting the validity of this TSS. In addition, these bases reside in the ~110-bp region identified by RT-PCR data. Primer PXR1B identified a unique start site at the GC residues in its ladder, which was overlooked by our RT-PCR experiment. Despite also terminating with a 5’ G residue, no stutter or doublet was observed so no conclusions about a capped or mature message can be made. Primer PXR1B extended through a proximal region containing other recognized start sites (Kurose et al., 2005), notably c* and d*. As illustrated in the additional sequencing panels of Fig. 2B, no termination product was seen in the region of start site either c* or d*. Results from primers PXR1A and PXR1B indicate two unique TSSs for the PXR 1 transcript.

Primer extension analysis of the PXR 2 transcript confirmed a TSS in the ~160-bp region between PCR primers PXR2A and PXR2.4. Two bands were generated by extension of primer PXR2A, which
terminated at C and T start sites separated by 3 nt. The upper C termination band appears to be more abundant than the lower T termination band; however, the 3-nt difference may be a product of experimental stuttering in a GC-rich region or a genuine wobble at the start of transcription. The primer PXR2B extension reaction also generated two termination products. The PXR2B-identified start sites are CC residues found 5 nt downstream from the start site(s) determined by primer PXR2A (Fig. 2D, top) and a G residue from a much shorter termination product (Fig. 2D, bottom). Unlike either of the PXR 1 primer extension primers, primer PXR2B is able to recognize more than one start site and in doing so enables us to evaluate those start sites based on abundance.

**Quantitation of PXR Isoform Expression by Real-Time PCR.**

The expression of multiple PXR isoforms that differ by first exons and promoter regions allows for a more specialized regulation and function of total PXR expression. Examining the isoform expression profile of our human liver samples will help to determine the importance of isoform PXR 2 with its unique first exon, distinct promoter, and additional N-terminal 39 amino acids. Previously, the PXR isoform profile was published from a larger study, but isoform PXR 2 was not included in that analysis (Lamba et al., 2004). Although on a smaller scale, our quantitative real-time PCR results reveal that total PXR expression varies widely by individual, but PXR 2, although significantly less abundant, maintains a consistent expression level (Fig. 3).

**Promoter Activation by PXR 1 and PXR 2.**

PXR 1 has been well characterized for its ability to induce CYP3A4 promoter activity. Other recognized isoforms have demonstrated a reduced capacity for ligand binding (Kliewer et al., 1998). However, initial characterization of PXR 2 suggests a comparable ability for transcriptional activ-
ity (Bertilsson et al., 1998). To investigate the activity of PXR 1 and PXR 2, HepG2 cells were transiently transfected with a luciferase reporter plasmid containing PXR-responsive elements from the distal and proximal regions of the CYP3A4 promoter and an expression plasmid for PXR 1 or PXR 2. In the response to the ligands rifampicin, RU486, and PBDE-71, both PXR 1 and PXR 2 significantly induced CYP3A4 promoter activity (Fig. 4). Tukey-Kramer honestly significant difference analysis showed no significant differences in CYP3A4 promoter activity between PXR 1 and PXR 2 expressing cells with these treatments. Cells treated with dexamethasone, cortisol, or lithocholic acid did not induce CYP3A4 promoter activity significantly over vehicle-treated controls. In these experiments, PXR 1 and PXR 2 showed comparable ability to activate transcription via interactions with the PXR-responsive CYP3A4 promoter.

**Discussion**

Years of research have proven that the orphan nuclear receptor, PXR, is critical to our ability to metabolize pharmaceuticals, interact with an environment rich in contaminants, and protect homeostatic systems from endogenous byproducts. The ligands and target genes of this xenosensing receptor have been well characterized, yet little research has been done to further understand the basics of PXR expression.

Since its initial discovery, PXR has existed in multiple transcript isoforms. PXR 2, with a unique 5' region and 39 additional N-terminal amino acids was discovered in tandem with PXR 1 (Bertilsson et al., 1998), and PXR 3, lacking a region of exon 5 in the ligand-binding domain, was characterized less than a year later (Dotzlaw et al., 1999). Despite the existence of these and other transcript variants, research on PXR function and its role in regulating metabolism has been largely limited to PXR 1. Alternate transcript isoforms are known to participate in the signaling of other nuclear receptors. For example, the progesterone receptor (PR, NR3C3) has two protein isoforms, PR-A and PR-B, and PR-B has a unique 164-amino acid N-terminal addition (Sartorius et al., 1994). Despite sharing all of the isoform PR-A sequence, the two receptor isoforms display different transactivation abilities, perform tissue-specific functions, and regulate distinct patterns of target genes (Sartorius et al., 1994; Mulac-Jericevic et al., 2000, 2003; Richer et al., 2002). PXR 2 originates from the same mechanisms of alternate processing as PR-B, whereas PXR 3 shares all sequences with PXR 1, differing only by a truncated ligand-binding domain. Here, on the basis of shared mechanisms, we suggest that isoform PXR 2 participates in the basic biology of PXR expression, on the basis of comparable function to PXR 1 and consistent mRNA abundance (Figs. 3 and 4).

Isoforms PXR 2 and PR-B share the unique feature of N-terminal addition compared with their alternate receptor isoforms. The N-terminus of nuclear receptors has not been well characterized. Unlike other receptor domains, the AF-1 domain and greater N-terminal region lack sequence identity across the receptor superfamilies but are similarly unfolded structures whose organization is greatly altered by interactions with other regions of the receptor. It has been suggested that variability in the N-terminal domain constitutes a possible determinant of receptor-specific responses through recruitment of various coactivators and corepressors (Li et al., 2003). PR-B contains 164 amino acids of N-terminal sequence that strikingly differentiates its function from that of PR-A, whereas PXR 2 has only 39 unique amino acids compared with PXR 1. Variant receptor isoforms with N-terminal additions are produced by the inclusion of a unique first exon. By definition, this unique first exon affords each transcript variant a distinct proximal promoter region to more specifically regulate its individual function (Landry et al., 2003). As demonstrated in the GR gene, the 5' region contains three separate first exons (denoted 1A, 1B, and 1C) and three independent promoters, one of which was recently discovered to be responsible for hormone-dependant up-regulation of the GR message (Breslin et al., 2001; Geng and Vedeckis, 2004). If GR and PR serve as examples for altered receptor isoforms, the N-terminal addition and proximal promoter of PXR 2 could greatly affect its expression and receptor biology.

The investigation of proximal promoters for PXR 1 and PXR 2 started with analysis of existing sequence information, both genomic and cDNA, and led to the discovery of a variety of TSSs. There was also a significant discrepancy in the recognized length of the 5' untranslated region (UTR) of each message. Because many of the designated TSSs were the product of cDNA library mining, we chose to experimentally determine a TSS for PXR 1 and PXR 2 from human liver tissue (Figs. 1 and 2).

The RT-PCR results mapping the 5' end of PXR 1 contradicted the start sites suggested by Zhang et al. (2001) and the NCBI reference sequence NM_003889. Kurose et al. (2005) published four TSSs for PXR 1 identified by 5' rapid amplification of cDNA ends, and our RT-PCR data contradicted those conclusions as well. Recognizing that RT-PCR would only definitively confirm the 5' most start site, primer extension experiments were used to more critically investigate the observed heterogeneity of the 5' UTR of PXR 1 (Fig. 5). Primer extension results, designed to account for all recognized start sites, identified two distinct TSSs for the PXR 1 message, one in agreement with the RT-PCR data (+112 at nt45535) and one further downstream (+115 at nt45777). Unfortunately, discovery of the two start sites using different primers prevents a quantitative evaluation. As shown in Fig. 2, our defined start sites reside in the vicinity of the a* and b* start sites identified by Kurose et al. (2005). In their 2005 publication, Kurose et al. concluded that the c* start site was the predominant PXR 1 TSS, labeling a*, b*, and d* as minor sites and eliminating them...
from further consideration. Despite the use of multiple primer extension primers, we failed to detect a termination product in the region of the c* start site. Based on a more precise technique and use of an RNA template, our primer extension data serve to refine existing TSS observations.

The mapping of the 5′ end of PXR 2 also began with contradicting the existing start sites from Zhang et al. (2001) and the NCBI reference sequence (NM_022002). Subsequent primer extension analysis of the PXR 2 transcript using the two primers, PXR2A and PXR2B, revealed two TSSs, one identified by each primer and one confirmed by both primers. The PXR2A-identified C and T sites and the PXR2B-identified CC site are 9 nt apart. The discrepancy between the two extension reactions can be attributed to the length of the PXR2B termination product, the GC-rich region, and the limited resolving power of the sequencing gel. On the basis of these considerations, we conclude that the C, T, and CC residues represent one PXR 2 TSS (+1235 at nt46159). The second PXR 2 TSS is identified by PXR2B as the G residue in its sequence ladder (+1238 at nt46403). The PXR2B-identified G residue is in the vicinity of the e* start site identified by Kurose et al. (2005), but our contention remains that primer extension has more accurately refined the PXR 2 TSS. The results of primer PXR2B, recognizing both start sites, allow us to conclude that the G termination product (+1238) represents the predominant TSS for PXR 2 based on abundance, and the C, T, or CC site serves as a minor start site for transcription.

Our new TSSs add as much as 300 bp to the 5′ UTR of PXR 1 and 120 bp to PXR 2, redefining the proximal promoters for each (Fig. 5). Our PXR 1 TSSs greatly differ from those commonly used in other promoter investigations, but little was known or investigated about PXR 2 TSSs. As defined by our new TSSs, 600 bp of proximal promoter for PXR 1 and PXR 2 were subjected to preliminary in silico analysis. Initial searches failed to find TATA binding sites in proximity to any of our three TSSs. However, genome-wide analysis of promoter regions has suggested that the importance of the TATA box to eukaryotic promoters has been overestimated. The less specifically defined initiator element and core promoter region have proven to be sufficient to recruit the necessary transcription factor complex (San-delin et al., 2007). Candidate response elements for liver-enriched factors such as hepatic nuclear factors 1 and 4, CCAAT/enhancer-binding protein family members, and other nuclear receptors such as PPAR and GR were found in the proximal promoters of both PXR 1 and PXR 2 (Heinemeyer et al., 1998). Additionally, binding sites for the transcriptional repressor, nuclear factor-xB were identified along with response elements for interleukins known to decrease PXR expression. On the basis of the nested organization of the PXR 1 and PXR 2 proximal promoters, the true determinant of expression may be the ability to interact with distal enhancer elements yet to be discovered.

The existing data on PXR regulation are limited. In experiments measuring expression, probes used to identify common regions of PXR isoforms complicate the ability to evaluate the induction by an individual isoform, whereas existing promoter studies are confounded by the use of different TSS and promoter regions. It was our own interest in the regulation of the multiple isoforms of PXR that led to the discovery of the 5′ UTR and TSS discrepancies and eventually to the data presented here. We have identified two TSSs for PXR 1 and two TSSs for PXR 2, none of which had been investigated directly from RNA. Also presented are data on the expression and transcriptional activity of PXR 2. Given the focus on PXR 1 in the current research, it is our contention that PXR 2 contributes to the overall function and basic biology of PXR expression and deserves further investigation.

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References


Breslin MB, Geng C-d, and Vedeckis WV (2001) Multiple promoters exist in the human GR gene, one of which is activated by glucocorticoids. Mol Endocrinol 15:1381–1395.


Gerbal-Chaînon S, Pascucci JM, Pichard-Garcia L, Dissanat M, Waechter F, Fabre JM, Carrere N,


Schuetz EG, Brimer C, and Schuetz JD (1998) Environmental xenobiotics and the antihormones cyproterone acetate and spironolactone use the nuclear hormone pregnenolone X receptor to activate the CYP3A23 hormone response element. Mol Pharmacol 54:1113–1117.


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