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**Unique Transcription Start Sites and Distinct Promoter Regions differentiate the
Pregnane X Receptor (PXR) isoforms PXR 1 and PXR 2**

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Running Title:

PXR 1 and PXR 2 have distinct proximal promoter regions

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Abbreviations:

PXR, pregnane X receptor

CYP3A4; cytochrome P450 3A4

5'UTR, 5' untranslated region

TSS, transcription start site

RT-PCR, reverse transcription-polymerase chain reaction

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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 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 PXR 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 PXR; yet PXR 2 expression represented a consistent 2-5% of total PXR expression, despite the observed variability. Transfection experiments demonstrated comparable ability of PXR 1 and PXR 2 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 PXR and should be included in the larger investigations of PXR expression and regulation.

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INTRODUCTION

The pregnane X receptor (PXR; NR1I2) was initially identified as an orphan receptor capable of binding the C21 steroid, pregnane and demonstrating the ability to regulate the promoter of monooxygenase cytochrome P450 3A4 (CYP3A4) (Bertilsson et al., 1998; Blumberg et al., 1998; Kliewer et al., 1998;). Since its discovery, significant research has been done to characterize this novel orphan receptor. PXR belongs to the nuclear receptor superfamily of genes. Like other family members, PXR behaves as a ligand-activated transcription factor, hetero-dimerizing with nuclear receptor RXR α (NR2B1) and recognizing response elements in the promoter of target genes to alter expression. Unlike other nuclear receptors, PXR contains a flexible ligand binding domain which allows for its unparalleled binding of structurally diverse endogenous and exogenous compounds (Watkins et al., 2001). Pharmaceutical drugs (glucocorticoids (Bertilsson et al., 1998), antivirals (Dussault et al., 2001), chemotherapeutics (Desai et al., 2002)), environmental pollutants (pesticides (Schuetz et al., 1998), phthalates (Takeshita et al., 2001), flame retardants (Pacyniak et al., 2007)), vitamins (Landes et al., 2003) and herbal supplements (Moore et al., 2000) have demonstrated the ability to bind PXR and activate the promoter of target genes. Endogenous compounds such as steroids (Kliewer et al., 1998) and bile acids (Staudinger et al., 2001) have also been identified as PXR ligands. In response to these ligands, PXR regulates numerous metabolic genes (CYP 2B, 2C, 3A (Lehmann et al., 1998; Gerbal-Chaloin et al., 2001; Goodwin et al., 2001); UGT (Gardner-Stephen et al., 2004), MDR1 (Geick et al., 2001; Synold et al., 2001). CYP3A4 is the phase I monooxygenase responsible for the metabolism of >60% of all pharmaceuticals and represents the most abundant CYP

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protein in the human liver (Li et al., 1995). The ability of PXR to regulate the expression of CYP3A4 has clinical implications for drug efficacy and the occurrence of adverse drug reactions.

Cytochrome P450 3A4 belongs to a small sub-set of CYP isoforms responsible for xenobiotic metabolism with a highly inducible expression profile. Many nuclear receptors, originally identified as orphans, are now recognized as the mediators of ligand-specific upregulation of a number of CYP isoforms. PXR, also identified as an orphan, was 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). Pascussi *et al.* (2001) suggested that the direct effect of GR was to upregulate 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 (FXR; NR1H4) (Jung et al., 2006), peroxisome proliferator activated receptor (PPAR; NR1C) (Aouabdi et al., 2006), and hepatocyte nuclear factor 4 (HNF4; 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

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expression have not been fully characterized. Transcript isoforms generated from alternative mRNA processing (splicing, exon usage, 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 most 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 (1998), Bertilsson (1998) and Blumberg (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 (TSS) for PXR 1 and PXR 2 by classical molecular methods and characterize each sample by its PXR isoform profile. Additionally, reporter assays using a CYP3A4 promoter construct demonstrated that PXR 2 has comparable transcriptional activity to PXR 1. In total, these data illustrate a

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need for better understanding of PXR regulation, isoform expression and the cumulative effect on the regulation of xenobiotic metabolism.

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MATERIALS AND METHODS

Human RNA Samples:

Whole human liver tissue samples (5-500 gm) were purchased from National Disease Research Interchange (NDRI, Philadelphia, PA). Tissue was snap frozen in liquid N₂ within 6-8 hours 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 ImpromII RT System (Promega, Madison, WI). PCR was done using the Hi-Fidelity PCR PLUS system (Roche, 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 (NCBI; <http://www.ncbi.nlm.nih.gov>) analysis was done 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 transcriptional start sites (TSSs) with two oligos (20- to 23-mer) for each transcript. For quantitative comparison, 10 pmol of each primer (see Table 1) was end-labeled with $\gamma^{32}\text{P}$ -ATP (sp. act. 3000 Ci/mmol) using T4 polynucleotide kinase according to manufacturer's instructions (Promega, Madison WI). After quenching with 0.5 M EDTA, 20 ug total

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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 AffinityScript Reverse Transcriptase (Stratagene, La Jolla, CA) under manufacturer's suggested conditions with the following modifications: 2.0 mM dNTP mix (0.5 mM each dNTP) and 15 U RNasin (Ribonuclease Inhibitor, Promega, Madison, WI). The reaction was incubated at 42°C for 30 min and quenched with 1 volume 2X loading dye (95% formamide; 18 mM EDTA; 0.025% SDS, xylene cyanol, and bromophenol blue). Each sample was heated to 90°C for 10 min before loading on sequencing gel.

A 2.5 kb genomic region (containing exon 1 of both PXR 1 and PXR 2) was TA cloned (Invitrogen, Carlsbad, CA) and used as template for the sequencing ladder. The 2.5 kb genomic construct was sequenced (SeqWright, Houston, TX) to ensure identity prior to use in any experiment. The sequencing ladder was generated with Sequenase version 2.0 Sequencing Kit (USB, Cleveland, OH). Products were separated on a denaturing 8% polyacrylamide sequencing gel (8 M urea). Images were captured with the Imaging Screen-K and the Molecular FX Imager (BioRad, Hercules, CA).

Plasmid constructs:

Expression constructs for PXR 1 (pcPXR1) and PXR 2 (pcPXR2) were generated by cloning the coding region for PXR 1 (amino acid 1-434; NM_003889) and PXR 2 (amino acid 1-473; NM_022002) into the pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA). The coding regions were amplified by PCR (as above) from HepG2 cell cDNA. The

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QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA) changed the divergent bases to match the respective reference sequences. The plasmid CYP3A4-luc, a kind gift from Dr. Jean-Marc Pascussi, contains the distal enhancer module (XREM) (-7836/-7208 nt) cloned 5' of the proximal promoter (-263/+11 nt) of the *CYP3A4* gene cloned into the pGL3 reporter vector (Promega, Madison, WI).

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 (Hs002543654_m1) and total PXR (Hs00243666_m1) and ribosomal 18S subunit were performed according to manufacturer's instructions.

Transient Transfection Assays:

HepG2 cells (human hepatoblastoma cells, ATCC, Manassas, VA) were plated at 3.0×10^5 cells per well. Twenty-four hours later, cells were transfected with 1.0 ug CYP3A4-luc and 0.1 ug empty vector pcDNA3.1(+), or pcPXR1, or pcPXR2 using TransIT-LT1 reagent (Mirus Bio Corp., Madison, WI) in Opti-Mem I reduced serum media (Invitrogen Corp.). Four to six hours later, media was removed and cells were allowed to recover overnight in Cellgro Complete media. Cells were treated for 48 hrs with vehicle control, DMSO and 10 uM rifampicin, 10 uM lithocholic acid (Sigma-Aldrich, St. Louis, MO), 10 nM dexamethasone, 10 uM RU486, 10 uM cortisol (Steraloids Newport, RI), or 10 uM polybrominated diphenyl ether-71 (PBDE-71) (A kind gift of Drs. Thomas Burka and J. Michael Sanders, NIEHS, RTP, NC). Following treatment, cells were lysed and

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harvested in 1X Reporter Lysis Buffer and processed according to Promega's Luciferase Assay System. 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 Tukey-Kramer HSD with a significance level of 0.05.

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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 transcriptional start site (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'RACE technique to explore an undetermined 5' region. Our forward primers were designed spanning 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 until 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 above, 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

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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. Based on 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 ~110bp 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 panel B, 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.

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Primer extension analysis of the PXR 2 transcript confirmed a TSS in the ~160 bp region between PCR primers PXR2.3 and PXR2.4. Two bands were generated by extension of primer PXR2A, which terminated at 'C' and 'T' start sites separated by 3nt. 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 5nt downstream from the start site(s) determined by primer PXR2A (upper panel, Fig 2D), and a 'G' residue from a much shorter termination product (lower panel, Fig 2D). 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 their analysis (Lamba et al., 2004). While on a smaller scale, our quantitative real-time PCR results reveal that total PXR expression varies widely by individual, but PXR 2, while significantly less abundant, maintains a consistent expression level (Fig 3).

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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 (Kliwer et al., 1998). However, initial characterization of PXR 2 suggests a comparable ability for transcriptional activity (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 (Rif), RU486, and polybrominated diphenyl ether-71 (PBDE), both PXR 1 and PXR 2 significantly induced CYP3A4 promoter activity (Fig 4). Tukey-Kramer HSD analysis found no significant differences in CYP34 promoter activity between PXR 1 and PXR 2 expressing cells with these treatments. Dexamethasone (Dex), Cortisol (Cort), and lithocholic acid (LCA) treated cells did not induce CYP3A4 promoter activity significantly over vehicle treated control. In these experiments, PXR 1 and PXR 2 illustrated 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

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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 (aa) 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, where PR-B has a unique 164 aa 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; Richer et al., 2002; Mulac-Jericevic et al., 2003). PXR 2 originates from the same mechanisms of alternate processing as PR-B, while PXR 3 shares all sequence with PXR 1 differing only by a truncated ligand binding domain. Here, based on shared mechanisms, we suggest that isoform PXR 2 participates in the basic biology of PXR expression, based on a comparable function to PXR 1 and consistent mRNA abundance (Figs 3 & 4).

Isoforms PXR 2 and PR-B share the unique feature of N-terminal addition compared to their alternate receptor isoforms. The N-terminus of nuclear receptors has not been well characterized. Unlike other receptor domains, the AF-1 domain and

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greater N-terminal region lack sequence identity across the receptor superfamily, 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 co-activators and co-repressors (Li et al., 2003). PR-B contains 164 aa of N-terminal sequence that strikingly differentiates its function from PR-A, while PXR 2 has only 39 unique aa compared to PXR 1. Variant receptor isoforms with N-terminal addition 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 3 separate first exons (denoted 1A, 1B, & 1C) and 3 independent promoters—one which was recently discovered to be responsible for hormone-dependant upregulation 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 impact 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. Since 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 & 2).

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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'RACE, 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 employed 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 2 distinct TSSs for the PXR 1 message—one in agreement with the RT-PCR data (+1^{1A} at nt45535) and one further downstream (+1^{1B} at nt45777). Unfortunately, discovery of the two start sites using different primers prevents a quantitative evaluation. As shown in Figure 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.* (2005) concluded the 'c*' start site to be 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 serves 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, discovered two TSS—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

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can be attributed the length of the PXR2B termination product, the GC-rich region and the limited resolving power of the sequencing gel. Based on these considerations, we conclude the 'C,' 'T,' and 'CC' residues represent one PXR 2 TSS (+1^{2A} at nt46159). The second PXR 2 TSS is identified by PXR2B as the 'G' residue in its sequence ladder (+1^{2B} 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 (+1^{2B}) 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, 600bp 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 (Inr) element and core promoter region have proven to be sufficient to recruit the necessary transcription factor complex (Sandelin *et al.*, 2007). Candidate response elements for liver-enriched factors such as HNF1 and HNF4, C/EBP family members, and other nuclear receptors such as PPAR and GR were found in the proximal promoters of both PXR 1 and PXR 2. Additionally, binding sites for the

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transcriptional repressor, NF- κ B were identified along with response elements for interleukins known to decrease PXR expression. Based 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 is limited. In experiments measuring expression, probes used to common regions of PXR isoforms complicate the ability to evaluate the induction by individual isoform; while 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 2 TSSs for PXR 1 and 2 TSSs for PXR 2, none of which had been investigated directly from RNA. Also presented is 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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Footnotes

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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.

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FIGURE LEGENDS

Figure 1. **RT-PCR mapping of PXR 1 and PXR 2 5' regions.** The schematic above illustrates the genomic organization of the first exons of PXR 1 and PXR 2 as described by Zhang et al. Sequence numbers below each exon correspond to sequence AF364606. Forward PCR primers are shown as arrows above and the PXR reverse primer (PXR RP) is shown below exon 2. The bent arrows indicate the regions where transcription start sites are likely to be found based on RT-PCR results. A) RT-PCR products from the 5' region of PXR 1 separated on an agarose gel. B) RT-PCR products from the 5' region of PXR 2 separated on an agarose gel. Data presented is a representative of PCR amplification with 5 individual human RNA samples.

MW=molecular weight marker; -DNA=no template control.

Figure 2. **Primer extension analysis of PXR 1 and PXR 2.** The schematic above illustrates the genomic organization of the first exons of PXR 1 and PXR 2 as described by Zhang et al. Sequence numbers below each exon correspond to sequence AF364606. Primers used for primer extension (horizontal arrows) are shown below and the transcription start sites reported by Kurose et al. (vertical arrows labeled with lower case letters and asterisks) are indicated above. In each panel, the corresponding DNA sequencing ladder is shown in the first 4 lanes and the extension product(s) are shown in the last lane. Sequences below each image are plus (+) sense; underlined bases indicate our mapped transcription start sites and asterisks (*) identify Kurose start sites labeled above. Primer extension reactions were performed with 20 ug of total RNA and

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10pmol end-labeled primer. Each primer extension product is from a single human RNA sample, but representative of the results from all five human samples. The results of each primer are presented in individual panels: A) primer PXR1A; B) primer PXR1B; C) primer PXR2A; and D) primer PXR2B. In panel D, adjacent termination sites determined by primer PXR2A are marked with a '^'.

Figure 3. Relative mRNA expression of PXR isoforms. The expression of all PXR isoforms and the specific expression of PXR 2 were measured in 6 human samples. A) The results of each TaqMan® assay are shown separately and B) by their contribution to total PXR expression (considered 100% for each individual).

Figure 4. Transcriptional activity of PXR 1 and PXR 2. HepG2 cells were transiently transfected with CYP3A4-luc and pcPXR1 or pcPXR2. Cells were treated for 48 hr with vehicle control (Ctl), 10 uM rifampicin (Rif), 10 uM lithocholic acid (LCA), 10 nM dexamethasone (Dex), 10 uM RU486, 10 uM cortisol (Cort), or 10 uM polybrominated diphenyl ether-71 (PBDE). Data is expressed as mean fold induction \pm SE. Statistical significance was determined by ANOVA, Dunnett's mean comparison test when compared to 'pcPXR1' vehicle control, * $p < 0.05$. This data set represents the average of three independent experiments, $n=9$.

Figure 5. New 5' map of the PXR gene. The schematic above illustrates the TSSs and proximal promoters for PXR 1 and PXR 2 based on our conclusions. Sequence numbers below each exon correspond to sequence AF364606. We have extended the

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PXR 1 5'UTR by ~300bp (+1^{1A}) or 60bp (+1^{1B}) and added ~120bp (+1^{2B}) to the 5'UTR of PXR 2. The alternate TSS for PXR 2 (+1^{2A}) is shown but considered to be a minor start site. Three receptor isoforms produced from our TSSs are shown below.

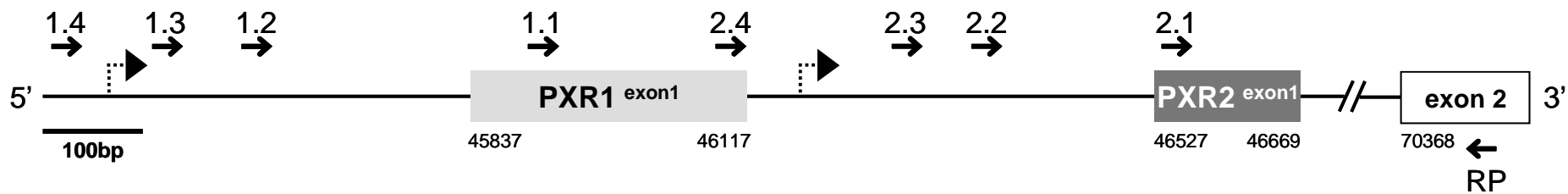
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Table 1. All oligonucleotide primers

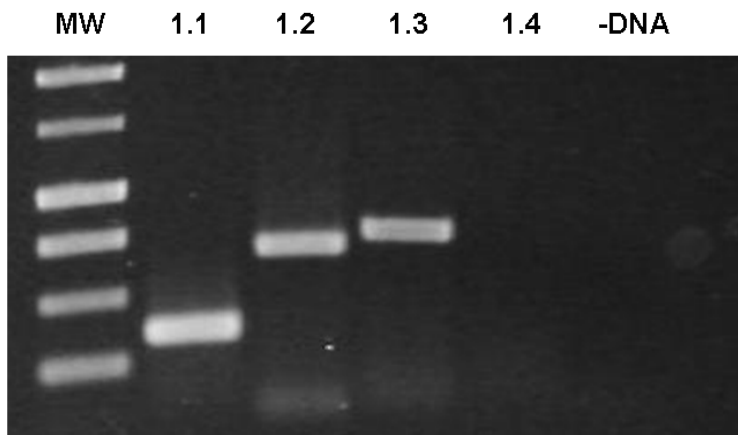
Name	Sequence (5' → 3')	Position ^a
PXR1.1	AGGACGGGAAGAGGAAGCA	45895
PXR1.2	GGTAAAGGACAGAGACCCTCA	45598
PXR1.3	CACTATCCAGGGAGGTGGTTTC	45524
PXR1.4	CATCTCACACCCTTTCCCTCTC	45415
PXR2.1	TTTGGCCTGCTGGGTTAG	46524
PXR2.2	CCTGCACTAGCCTCCTAGAAAAA	46338
PXR2.3	GTCTCCTCATTCTAGGGTGA	46257
PXR2.4	GGGAGAAGTCGGAGCAAAGA	46082
PXR RP	GAAGTCGGAGGTCCCCAAATCT	70510
PXR1A	AGGAAAAGGGGAAGAGAGTGGC	45686
PXR1B	TGCCTTTTTTCAGGACAGGAG	46015
PXR2A	TTTTTCTAGGAGGCTAGTGCAGG	46360
PXR2B	CTTGGAGGTGGTATTTCTG	46470

^aPosition numbers based on genomic sequence AF364606.

Figure 1



A



B

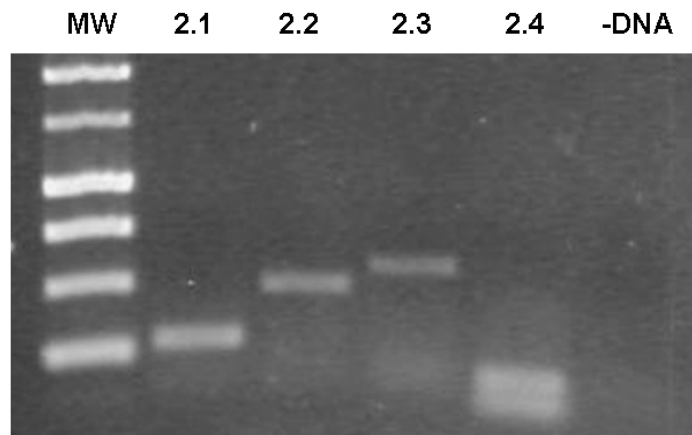


Figure 2

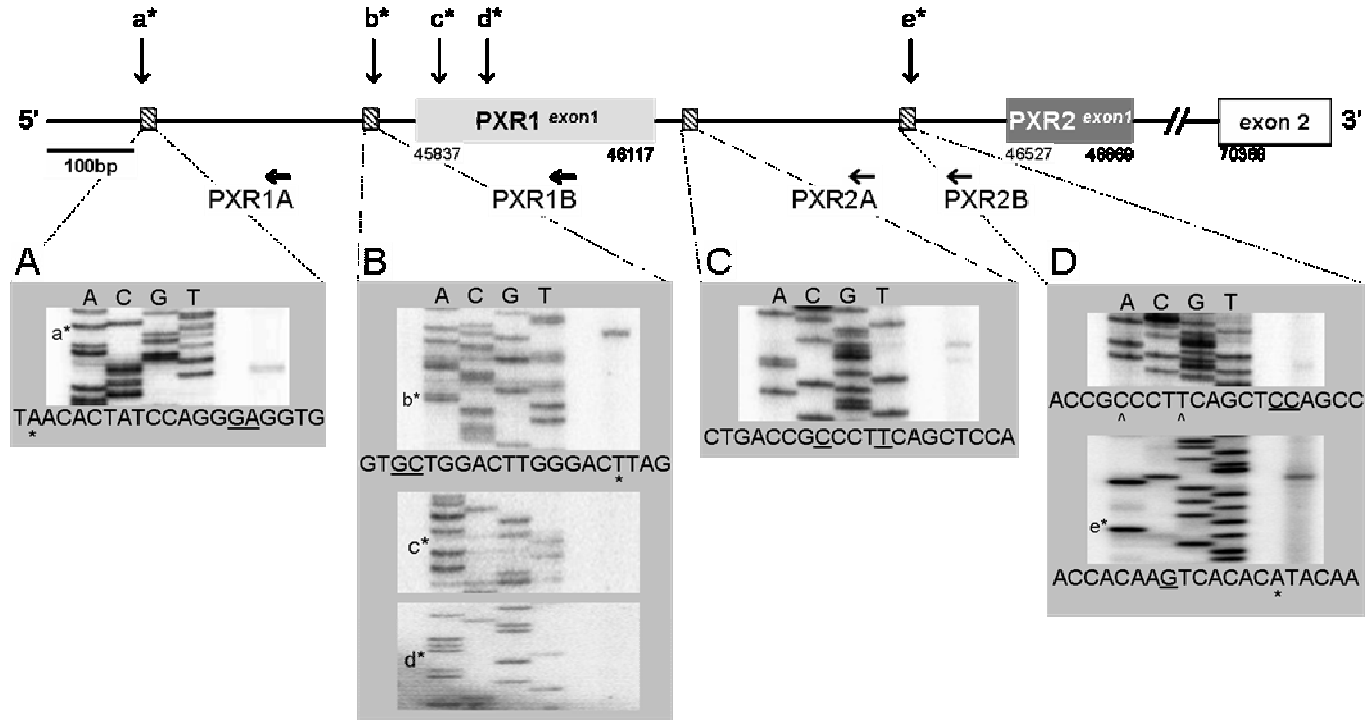


Figure 3A

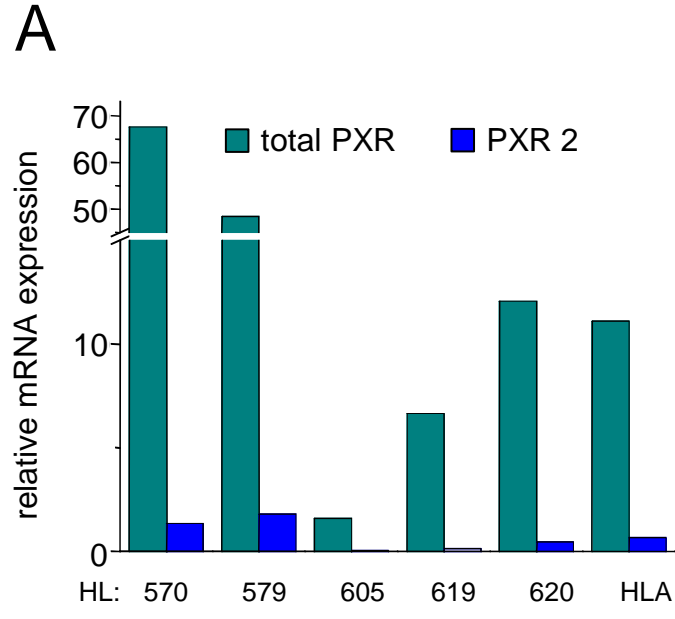


Figure 3B

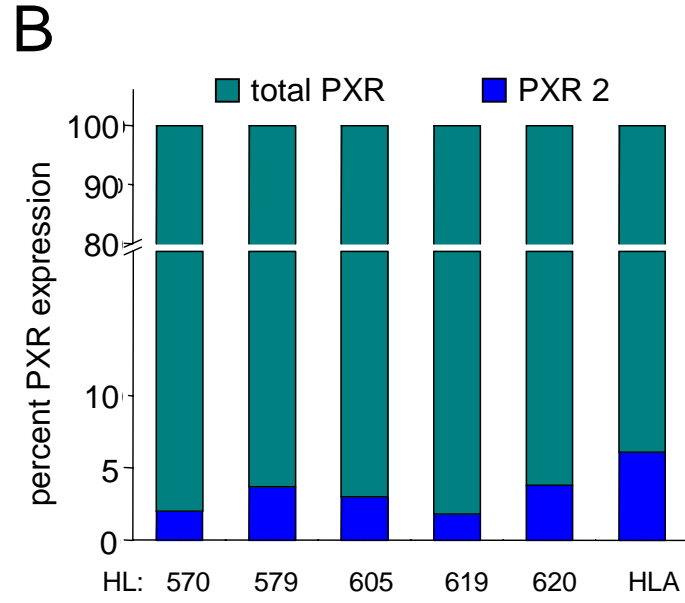


Figure 4

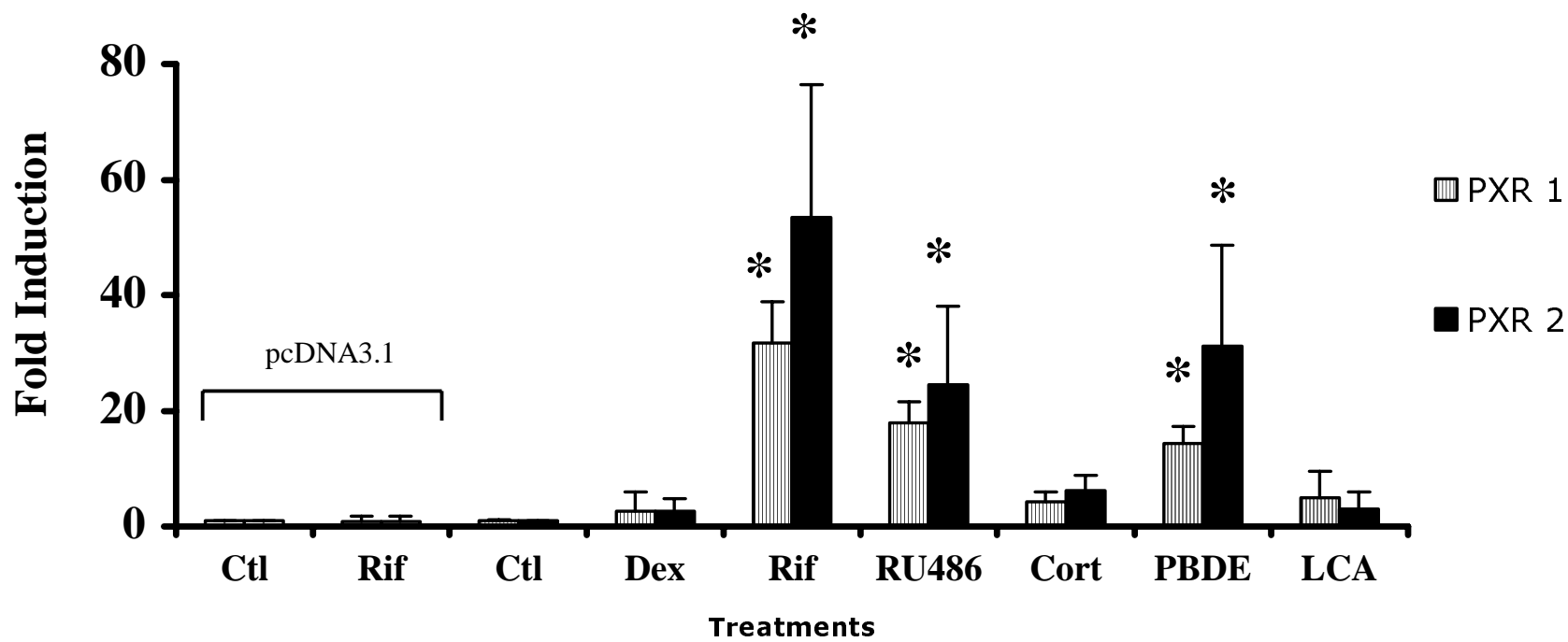


Figure 5

