ABSTRACT

TOMPKINS, LESLIE MEREDITH. Identification of Novel Transcription Start Sites and Alternative Proximal Promoters for Pregnane X Receptor (PXR) Isoforms PXR 1 and PXR 2. (Under the direction of Dr. Robert C. Smart and Dr. Andrew D. Wallace.)

The identification and characterization of the orphan group of nuclear receptors has revolutionized our collective understanding of signaling pathways responsible for xenobiotic metabolism. Discovery of the pregnane X receptor (PXR) provided the mechanistic explanation to decades of research that documented xenobiotic metabolic regulation and hepatic protection through enzyme induction without a known mediator. PXR, known as the xenosensing receptor, coordinately regulates 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 itself are few. After an initial review of available sequence data, we discovered discrepancies in the 5' untranslated region (UTR) and transcription start site (TSS) characterizations of the PXR gene, so we endeavored to define TSSs and proximal promoters for the well-known PXR 1 isoform and the lesser-known PXR 2 isoform. The PXR 2 isoform is the product of a unique first exon responsible for the 39 amino acid amino-terminal addition and is therefore regulated by a distinct proximal promoter. RT-PCR and primer extension experiments performed using total RNA from human liver samples identified two TSSs for each receptor isoform. These results extended the 5'UTR
sequence of each isoform and defined new proximal promoters for both PXR isoforms. Separate promoter regions for receptor isoforms suggests the possibility that PXR isoforms are differential regulated or expressed in a tissue-specific fashion. Candidate response elements for liver-enriched transcription factors and other nuclear receptors were found in both promoters. Quantitative PCR illustrated PXR 2 expression as a modest but consistent 2-5% of total PXR expression with much less variability among individuals. In addition, transfection experiments demonstrated comparable transcriptional activity of PXR 2 when compared to that of PXR 1. In total, these data present a starting point from which to investigate the regulation of multiple isoforms of PXR and evaluate individual isoform contributions to the cumulative function of this important nuclear receptor in xenobiotic metabolism.
Identification of Novel Transcription Start Sites and Distinct Proximal Promoter Regions for Pregnane X Receptor (PXR) Isoforms PXR 1 and PXR 2

by

Leslie Meredith Tompkins

A dissertation submitted to the Graduate Faculty of North Carolina State University
In partial fulfillment of the Requirements for the degree of Doctor of Philosophy

Toxicology

Raleigh, NC
2007

Approved by:

Dr. Robert C. Smart
(Co-chair of Advisory Committee)

Dr. Andrew D. Wallace
(Co-chair of Advisory Committee)

Dr. William Miller

Dr. Ernest Hodgson
DEDICATION

I dedicate this work to…

My Mom & Dad,
without their love and support, none of this would have been possible.
Everything I am, I owe to them.
I love you.

And

Mr. Charles Fisher and Mr. Leo Kelley,
who introduced me to the world of science and inspired this journey.
It’s a gift I’ll treasure for the rest of my life
I am Leslie Meredith Tompkins, daughter of Sharon St.Cyr and Howard Burton Tompkins of Bordentown, New Jersey. Born and raised in a small town, our family moved into my one and only home when I was a little more than a year old and my parents still live there to this day. It provided me with everything I needed—food in the refrigerator, cable TV in the living room, my own bedroom and a pool in the backyard. I spent all of my first 18 years there and graduated from Bordentown Regional High School in 1995.

My mother is a vocal music teacher for elementary children in a neighboring town and my father works for the state’s Department of Environmental Protection. They are both college graduates and professionals in their respective fields. I don’t remember a time in my life when it wasn’t expected that I would go to college. My mother has a Bachelor’s of Fine Arts in Music Education from Michigan State University and my father, a Master’s of Public Health from the University of Michigan. Not only did I get life lessons from my parents, they also taught me about Big Ten rivalry!

My parents are high school sweethearts from a small town in upstate New York who left home for college to find an independent life and did so together. They were, individually, among the first generation in their families to go to college and they were also first to make the choice not to build a life in the town they grew up in. They desired a life beyond their small town, a life of their own making and on their
own terms. This is the desire they instilled in me. I remember being encouraged from a very young age to be independent and find my own way. As a rambunctious 3 year old, my mom dropped me off for my very first day of all-day preschool fully expecting the tears and tantrums she saw every first day of school; instead I eagerly walked through the door and turned around with a casual wave and said, “Bye, Mom.” It was then that they knew their only child was ready to take on the world.

My college years took me to coastal New England and the University of New Hampshire. We spent many winter and summer family vacations in the New England area which inspired my choice of university. Despite the disapproving look from my dad because UNH was simply too far away, I knew the moment I stepped on campus this was where I belonged. I knew science would be my major but I knew little else other than I wanted to make the most of my college experience.

While at UNH: I became a Biology major; I discovered NCAA college hockey; I joined the university concert choir; I survived hours of lectures and labs and continued to learn more about myself with each additional class I took. Best of all, second semester junior year, I had the privilege to study abroad in Australia at Griffith University in Brisbane, QLD. I can honestly say the decision to go to Australia was the best decision I’ve made in my life, so far. Academically, it was a break from the rigors of a demanding science curriculum at UNH, and personally, it was an opportunity to reinvent myself, make new friends and explore. This 5 month experience opened my eyes to everything beyond my small hometown and college lifestyle and changed my perspective. This adventure also took me 14,000 miles
away from everything I knew in the world, much to the concern of my parents, but they raised an independent daughter and that’s exactly what they got. Despite their concerns, they fully supported my travels half way around the world and even managed to plan an Australian vacation for themselves.

I returned from my semester in Australia with a renewed dedication to learning and science. With a new awareness of how big the world was and how much there was to learn, I decided to plan for graduate school and continue my education. The planning and deciding on graduate school takes time and research, but I was still readjusting to traditional American college life and taking a full semester’s worth of classes. Fortunately for me, with the Peterson’s Guide to Graduate Programs in hand, my dad and I became a team sifting through the hundreds of programs and options before me. It was my dad who found the Toxicology Department at North Carolina State University and encouraged me to apply. It was the last application I sent and I did so simply because he asked me to. I was accepted into the program on my merits and qualifications, but my dad was the single most important factor in getting me there.

The rest, as they say, is history. My graduate experience has been quite a journey—not at all what I expected when I first arrived in August 1999 as a very young 22 year old. I have benefited from my interactions with faculty and dedicated mentors and learned from my failures. As for the future, it is still to be determined, but I’ll have this degree, this graduate experience, and everything I’ve learned and carry it with me always.
ACKNOWLEDGMENTS

I would like to thank my advisor and committee members: Drs. Andrew Wallace, Robert Smart, William Miller and Ernest Hodgson for their participation and guidance. I think it’s fair to say this hasn’t been an average committee experience for any of the committee members involved. I have asked for significant committee involvement in the last year or so and all four men have willingly dedicated their time and energy to me. For all that they’ve done, I am exceedingly grateful.

I am eternally and forever indebted to Drs. Donna Newman and Tim Sit, who gave of themselves and helped to make me the scientist I am today. As a very green and inexperienced graduate student, Donna took me under her wing and guided me through the basics of laboratory science. She became everything from mentor, brainstorming partner, and confidant even to surrogate mom. She gave me the basis from which everything eventually grew. Tim was an upstairs neighbor in a lab from another department that no one knew very well, but I would quickly discover his lab bench was a popular destination for lost graduate souls, like me. Experimental crisis led me to seek Tim’s assistance and what I received was the most significant mentoring experience in my graduate career. Despite having no affiliation or responsibility for me, he gave of his time and his energy, and most special to me, his friendship.
My graduate career wouldn’t be the same without the people and the friends that give this department life. I have made a number of friendships over the years, and while some are still here and some are gone, they’re all special to me.
TABLE OF CONTENTS

List of Figures  --------------------------------------------------------- ix
List of Tables  --------------------------------------------------------- x

INTRODUCTION  --------------------------------------------------------- 1
  1. Nuclear Receptors  ------------------------------------------------- 2
  2. PXR and Xenobiotic Metabolism  ------------------------------------- 12
  3. NR1I2; the Pregnane X Receptor, PXR  ----------------------------- 20
  4. Hypothesis and Rationale  ----------------------------------------- 27
  5. References  -------------------------------------------------------- 33

UNIQUE TRANSCRIPTION START SITES AND DISTINCT PROMOTER REGIONS
DIFFERENTIATE THE PREGNANE X RECEPTOR (PXR) ISOFORMS PXR 1 AND
PXR 2  ------------------------------------------------------------- 47
  1. Abstract  --------------------------------------------------------- 47
  2. Introduction  ------------------------------------------------------ 48
  3. Materials and Methods  -------------------------------------------- 51
  4. Results  ---------------------------------------------------------- 55
  5. Discussion  -------------------------------------------------------- 58
  6. References  -------------------------------------------------------- 72

CONCLUSION  ----------------------------------------------------------- 79
  References  ---------------------------------------------------------- 83

APPENDIX  ------------------------------------------------------------- 86

  Tompkins, L M and Wallace, A D., Mechanisms of Cytochrome P450
LIST OF FIGURES

INTRODUCTION
Figure 1. Common structural domains of nuclear receptors 30
Figure 2. Structural variety of PXR ligands 31
Figure 3. Diagram of human PXR isoforms, PXR 1, PXR 2 & PXR 3 32

UNIQUE TRANSCRIPTION START SITES AND DISTINCT PROMOTER REGIONS DIFFERENTIATE THE PREGNANE X RECEPTOR (PXR) ISOFORMS PXR 1 AND PXR 2
Figure 1. RT-PCR mapping of PXR 1 and PXR 2 5’ regions 66
Figure 2. Primer extension analysis of PXR 1 and PXR 2 67
Figure 3. Relative mRNA expression of PXR isoforms 69
Figure 4. Transcriptional activity of PXR 1 and PXR 2 70
Figure 5. New 5’ map of the PXR gene 71

APPENDIX
Figure 1. PXR Mechanism of Action 97
LIST OF TABLES

UNIQUE TRANSCRIPTION START SITES AND DISTINCT PROMOTER REGIONS DIFFERENTIATE THE PREGNANE X RECEPTOR (PXR) ISOFORMS PXR 1 AND PXR 2
   Table 1. All oligonucleotide primers ----------------------------------------------- 65

APPENDIX
   Table 1. Inducible CYP isoforms ----------------------------------------------- 96
INTRODUCTION

The pregnane X receptor (PXR, NR1I2) belongs to a superfamily of ligand-activated transcription factors responsible for regulating a variety of functions throughout the body. Originally characterized as an orphan receptor, PXR has since demonstrated the ability to regulate the expression of xenobiotic metabolizing genes in response to a broad range of ligands. Most notably, the regulation of cytochrome P450 3A4 (CYP3A4) expression has made PXR an important participant in the regulation of metabolism [1-3]. CYP3A4 is the most abundant CYP protein in the human liver and a member of a small sub-set of CYP isoforms responsible for xenobiotic metabolism. Capable of metabolizing greater than 60% of all pharmaceuticals, CYP3A4 is also highly inducible by an equally broad range of xenobiotics mediated through PXR binding and transcriptional activation [4]. The ability of PXR to regulate the expression of CYP3A4 has clinical implications for drug efficacy and the occurrence of adverse drug reactions. Thus, understanding the biology of this unique orphan receptor will further our understanding of xenobiotic metabolism.

Little is known about PXR expression and regulation. While predominantly expressed in liver and small intestine [1, 2, 5], PXR message has been detected in a number of tissues including lung [6] and brain [7]. In addition, PXR expression consists of multiple transcript isoforms generated by alternative splicing and alternate promoter usage [1, 7-9]. In large part, the biology of these alternate
isoforms is unknown. Published research on the regulation of PXR has suggested that other nuclear receptors, such as glucocorticoid receptor (GR, NR3C1) [10] and hepatocyte nuclear factor 4 (HNF-4, NR2A1) [11] may participate in PXR promoter activation. The ability of other nuclear receptors to alter PXR expression adds additional complexity to the regulation of this critical signaling pathway. These findings, along with available DNA sequence information, were used to propose a hypothesis: differential regulation of alternate receptor isoforms of PXR with distinct promoter regions allows for a more specialized function of alternatively spliced receptor isoforms. Our experimental design focused on characterizing transcription start sites and proximal promoter regions for PXR isoform 1 (PXR 1) and PXR isoform 2 (PXR 2). The results of this investigation are presented in the manuscript that follows. Preceding the manuscript is a review of nuclear receptors and xenobiotic metabolism focusing on PXR.

1. Nuclear Receptors

The nuclear receptor superfamily

The existence of high-affinity receptors mediating steroidal activity has been known for more than 20 years, but cloning of the first nuclear receptor wasn’t accomplished until 1985 [12-16]. In the years that followed, more steroid receptors were cloned and additional receptors were discovered by their shared identity to known receptors sequences. Today, sequencing of the human genome has identified 48 nuclear receptors that constitute the superfamily of ligand-activated
transcription factor genes. Based on sequence identity, the 48 receptors have been classified into 6 evolutionary groups (NR1-NR6) [17, 18]. However, based on source and type of endogenous ligand, nuclear receptors fall into only three categories: 1) steroid or endocrine receptors; 2) adopted orphan receptors; and 3) true orphan receptors [19]. The steroid receptor group consists of receptors for endogenous hormones such as estrogen (ER, NR3A1), progesterone (PR, NR3C3), and thyroid hormone (TR, NR1A1). The cellular activities of each steroid ligand were well documented and the steroid receptors were discovered as the mediators of that activity. On the contrary, orphan receptors were discovered without ligand or function. Through the benefit of new molecular techniques and libraries of sequence data, new receptors were identified by their similarity to existing receptors with their purpose and function to be determined later. Those receptors for which an endogenous ligand has been identified and a function has been determined are referred to as ‘adopted.’ The pregnane X receptor (PXR) is an ‘adopted’ orphan. Many of the identified nuclear receptors still remain in the true orphan category.

**DNA binding and DNA response elements**

Common to all nuclear receptors, with two minor exceptions, is their ability to dimerize in response to ligand binding and bind sequence-specific DNA response elements in the promoter of regulated genes. Type I steroid receptors, represented by GR, PR, ER, mineralcorticoid (MR, NR3C2), and androgen (AR, NR3C4) receptors, homodimerize and bind palindromic repeats, while the adopted orphans
and type II steroid receptors (such as vitamin D receptor (VDR, NR1I1) and retinoic acid receptor (RAR, NR1B1)) heterodimerize with a common partner, retinoid X receptor-α (RXRα, NR2B1) and bind imperfect bipartite sequences with spacer nucleotides. The organization of the nuclear receptor binding site, whether direct (DR), inverted (IR) or everted (ER) repeats, and the number of nucleotide spacers determines the specificity of binding for heterodimer partners. The PXR/RXRα heterodimer pair is capable of binding a direct repeat with a 3nt spacer (DR3), DR4, and everted repeat with a 6nt spacer (ER6) response elements in the promoters of a variety of xenobiotic genes [5, 20]. It is possible for other RXR-heterodimer pairs to bind DR4 or ER6 elements [21]; however the folded receptor pair also has a preference for nucleotides adjacent to the repeated sequence adding to response element specificity.

The principle function of nuclear receptors is to recruit necessary cofactors and the transcriptional machinery to increase target gene expression. The necessary and nonredundant roles for nuclear receptors in the regulation of growth and development, and metabolism and homeostasis are not limited to transcriptional gene activation. Thyroid hormone receptor (TR) and RAR exhibit a ligand-independent ability to repress target gene expression through interaction with nuclear receptor corepressor, NCoR, and silencing mediator for retinoid and thyroid hormone receptors, SMRT [22, 23]. Distinct from transcription-dependent function, nongenomic activities of nuclear receptors have recently been recognized—such as GR demonstrating an ability to interfere with the signaling of other transcription
factors, such as AP-1 and NF-κB [24, 25] and steroid receptors being implicated in altering the concentration of intracellular second messengers such as Ca$^{2+}$ and cAMP [26, 27]. As a family, the function of nuclear receptors is the cumulative outcome of a variety of complex mechanisms.

**Nuclear receptor structure**

Despite functional or evolutionary differences, all nuclear receptors share the same basic structural domains. As shown in Figure 1, each nuclear receptor contains five structural domains: N-terminal region (A/B), DNA-binding domain (C), hinge domain (D), ligand binding domain (E) and C-terminal region (F) [28].

The DNA-binding domain (DBD, region C) is the most conserved region across the nuclear receptor superfamily. It contains a 66-residue conserved core made up of 2 zinc-finger domains, 2 α-helices and a C-terminal extension [29]. Specific residues within each region have been identified that house the traditional functions attributed to the DBD. Within the N-terminal α-helix (helix 1), the P-box is made of residues that define the specific receptor-DNA response element interaction [30-32]. The second zinc finger and C-terminal extension house a T-box and an A-box which undergo helical ordering necessary for DNA binding of a RXR heterodimer. The T-box facilitates the dimer interface with RXR’s second zinc finger, while the A-box directly contacts the DNA [29, 33]. The DBD can also participate in nuclear localization and cofactor binding [34].
The ligand binding domain (LBD, region E) is a functionally complex region containing the ligand binding pocket, surfaces for dimer and cofactor binding and the ligand-dependent activation function, AF-2 domain. RXR$_{\alpha}$ was the first receptor to demonstrate the antiparallel ‘$\alpha$-helical sandwich’ structure found in all nuclear receptors [35]. The LBDs 12 $\alpha$-helices and short $\beta$-turns are arranged in three layers constituting two outer faces and a central layer. Comparison of LBDs across the receptor superfamily has identified regions of structural similarity excluding the variable ligand binding pocket [36, 37]. Located within the structure, the ligand binding pocket is defined by the surrounding helices and protruding polar groups. The LBD/ligand complex is largely determined by hydrogen bonding and hydrophobic interactions, while being restricted by the steric size and shape of the ligand binding pocket itself. Having high affinity interactions with a smaller number of ligands, the small volume of the steroid receptor ligand binding pocket correlates with a more restricted ligand profile [38]. For example, GR, responsible for binding structurally similar endogenous and therapeutic glucocorticoids, has a ligand binding pocket volume of 578 to 599 Å$^3$ [39]. The orphan receptors bind a diverse array of metabolic ligands with lower affinity and therefore, exhibit significantly larger ligand binding pocket volumes. PXR, representing the orphan receptors, demonstrated a ligand binding pocket volume of 1294-1544 Å$^3$ [40]. The completion of the PXR LBD crystal structure allowed for a physical explanation of its observed promiscuous ligand binding behavior. Unique to PXR are 1) a variable region in place of $\alpha$-helix 2 proposed as a ligand access site; 2) replacement of $\alpha$-helix 6 with a flexible loop
adjacent to the ligand binding pocket allowing for the accommodation of larger molecules; and 3) two additional β strands forming a five-stranded antiparallel β-sheet previously unseen in nuclear receptor LBD structure. The PXR ligand binding pocket itself is characterized by 28 amino-acid side chains, only eight of which participate in hydrogen bonding with ligands [41]. All unique attributes of this orphan nuclear receptor contribute to its ability to bind ligands of varying size and structure.

While ligand binding is the first step in receptor activation, receptor dimerization and cofactor binding are also necessary functions of the LBD in order to complete receptor function. The crystal structure of steroid receptor homodimers and RXR-orphan heterodimers demonstrate shared structural elements at the dimerization interface resulting in symmetric homodimers and slightly asymmetric heterodimers [36, 37]. Ligand-dependent effects on RXR heterodimers suggest an interaction of the ligand binding pocket and the components of the dimerization interface [42, 43]. Once formed, nuclear receptor dimers recruit cofactors and activators of transcription via the ligand-dependent activation function, AF-2 domain. Localized to α-helix 12, renamed α AF-2, the conserved α-helical LxxLL motif within is recognized by members of the p160 family of transcriptional activators, such as SRC-1 and GRIP-1 [44-46]. Ligand binding and subsequent receptor dimerization causes a conformation change, a repositioning α AF-2 and generation of a transcriptionally competent receptor complex [38]. Subsequently, histone
acetyltransferases (HATs) and the RNA polymerase II holoenzyme assemble at the site of p160 family member recruitment to initiate transcription [47].

The remaining structural domains: the N-terminal region (A/B), the hinge domain (D) and the C-terminal region (F) are considerably variable across receptor sequences and not well characterized with respect to function. The C-terminal F region, without a defined function, is contiguous with the LBD, region E and not found in all nuclear receptors. The hinge domain, region D, is located between the DBD and LBD and its primary function is allowing the receptor to adopt a proper tertiary structure without steric hindrance. There are also elements of a nuclear localization signal and sites for phosphorylation that are suggestive of other accessory, if not independent, roles for this region once thought to be functionally inert [17]. The N-terminal region (A/B) contains the ligand-independent activation function, AF-1 domain. Found in all nuclear receptors, the AF-1 domain has the autonomous ability to activate transcription in the absence of the LBD, but in the context of a full-length receptor its activity appears to be ligand-dependent. In addition, the N-terminal AF-1 domain has also demonstrated the ability to recruit transcriptional cofactors such as SRC-1 [48]. The structure of the N-terminal region varies widely with a considerable lack of conservation across receptors. However, it has been suggested that this variability in N-terminal sequence allows for receptor-specific differences in coactivator recruitment [49].
All parts of the receptor have an individual function and can be analyzed separately, but it’s the interaction of the holoprotein that determines the functional outcome of receptor activation.

Expression and receptor isoforms

Nuclear receptors participate in a variety of critical functions in every tissue in the body. Their ligand-activated functions have important roles in regulating development, physiology, proliferation and metabolism. Therefore, the expression of each nuclear receptor is one determinant of the outcome of any specific receptor signaling pathway. In addition to the amount of expressed receptor, the variety of expressed receptors, also known as receptors isoforms, affects the functional outcome of receptor activation. Some receptors exist as subtypes or isotypes (RARα/RARβ/RARγ) which are the products of related but independent genes. They demonstrate unique and redundant roles in their combined regulatory function. Other receptors are expressed as multiple receptor isoforms that are the products of a single gene but generated through alternative splicing, alternative promoter and exon usage, and alternate translation initiation. For example, GR is expressed as GRα and GRβ [12], PR is expressed as PR-A and PR-B [50], and PXR is expressed as PXR 1, PXR 2, PXR 3 and other documented isoforms [1, 7-9].

Completion of the human genome sequencing identified an approximate total of 20,000 – 25,000 human genes, a number much smaller than expected based on the known variety of human protein expression. Based on the ‘one gene, one
protein’ assumption and the size of the human genome, scientists predicted finding 60,000 to 100,000 genes. Lesser recognized theories of alternate mRNA processing mechanisms began to get more attention for their ability to generate a variety of protein isoforms from a single gene. Today, it is estimated that more than 50% of all human genes have multiple mRNA products produced from alternate mRNA processing [51]. The original discovery of GR included the identification of GRα and GRβ, which differed only by the inclusion of an alternate ninth and final exon [12]. The PR and PXR both have additional isoforms (PR-B and PXR 2) generated by usage of a unique first exon [1, 50]. Substitution at the first exon also implies the presence of an alternate promoter directly 5’ prime of the new first exon. The use of alternate promoters has a number of outcomes: 1) no change in the receptor isoform, based on translation initiation in a common downstream exon, but variable 5' regions allow for tissue-specific expression or altered translation efficiency; 2) alternate 5’ exons may contain additional ATGs resulting in a protein isoform that differs by N-terminal addition and 3) addition of 5’ regions may introduce a new open reading frame (ORF) and translate an entirely different protein [52]. Both PR-B and PXR 2 are products of N-terminal addition. Research has demonstrated that PR-B has 164 amino acids of additional N-terminal sequence, within which is contained a unique third activation function, AF-3 and the unique PR-B promoter region causes distinct tissue-specific expression compared with PR-A [53-55]. PXR 2 is known to contain 39 additional N-terminal amino acids, but little else is known about its impact on isoform function or the significance of its
alternate promoter. Discovery of isoform-specific functions, whether for GRβ or PR-B, illustrate the functional importance of alternative receptor isoforms.

**Nuclear Receptor Function**

As a family of related proteins, all nuclear receptors have relatively the same requirements for receptor activation and subsequent transcription of a given target gene. However, evaluation of these specific requirements for each receptor revealed two distinct paradigms of receptor function [19]. The distinction occurs between traditional steroid/endocrine receptors and the orphan/RXR heterodimer receptors. The steroid receptors, including GR, PR, ER, MR, and AR, bind (predominantly) endogenous ligands from an endocrine source with very high affinity. These receptors have critical functions in sexual differentiation, reproduction and development and their function is strictly regulated by negative-feedback controls for receptor expression and ligand availability via the hypothalamic-pituitary axis [56]. On the other hand, orphan receptors, such as PXR, PPAR, liver X (LXR, NR1H3) and farnasoid X (FXR, NR1H4) receptors represent the second receptor paradigm. These receptors bind a variety of dietary lipids, bile acids, oxysterols, fatty acids and xenobiotics with relatively low affinity and function to activate a feed-forward cycle of metabolic genes—suggesting a purpose as ‘sensor’ receptors. There is no feedback mechanism for dietary or pharmaceutical ligands and information on orphan receptor regulation is limited. The outcome of receptor
activation is far-reaching with target genes participating in metabolism, storage, transport and elimination of each specific ligand [19].

In total, the superfamily of nuclear receptor proteins, while related in structure and similar in behavior, varies widely in function.

2. PXR and xenobiotic metabolism

Cytochrome P450s

The family of heme-binding monooxygenases, the cytochrome P450s (CYPs) are responsible for the metabolism of endogenous and exogenous compounds. CYP proteins, representing phase I in the metabolic system, modify lipophilic substrates by attaching a hydroxyl group that will subsequently be used by phase II conjugating enzymes for further modification. Conjugated metabolites of endogenous and exogenous compounds alike are removed from the cell by transport proteins, sometimes known as phase III enzymes [57, 58]. This tri-phasic pathway functions to regulate intracellular levels of compounds and metabolite(s) protecting against toxic accumulation.

In humans, there are 57 known CYP proteins that encompass a wide variety of function and display significant differences in substrate specificity, but only a few family members are dedicated to xenobiotic metabolism [59]. Members of the CYP1A, CYP2B, CYP2C, CYP3A and CYP4A families have a preference for xenobiotic substrates and are distinguished by their highly inducible expression profile [60]. Expressed at relatively low, basal levels in the absence of substrate, the
dramatic increase in CYP expression mediated by xenobiotics provides for an amplified metabolic response following xenobiotic exposure. Most notable among the xenobiotic metabolizing CYPs are the 3A family members—CYP3A4, the dominant CYP3A isoform in humans, represents the most abundant CYP protein in human liver. Combined with the induction by a structurally diverse group of xenobiotics and the ability to metabolize 50-60% of all pharmaceuticals currently in use [61, 62], the expression and induction of CYP3A4 represent a mechanism for altered endogenous metabolism and adverse drug reactions. The observation of ‘catatonic steroids’ and their ability to upregulate CYP3A expression and induce monooxygenase activity was made more than 40 years ago [63], but the question that remained was how small, lipophilic compounds could affect the regulation of specific CYP isoforms. Compounds such as phenobarbital (PB) and rifampicin (Rif) demonstrated an ability to induce the expression of CYP2A, CYP2B, CYP2C, and CYP3A with each xenobiotic representing its own profile of induction based on varying potencies for individual CYP isoforms [64-66]. For example, rifampicin more efficiently induced CYP3A expression while phenobarbital predominantly activated transcription of the CYP2Bs and CYP2Cs [65]. These two xenobiotics became representatives of a class/group of substrates capable of regulating the same profile of genes, i.e. PB-like inducers or Rif-like inducers. Not limited to the induction of CYP isoforms, the transcriptional activation by these xenobiotics extended to phase II conjugating enzyme and transporter proteins resulting in a pleotropic acceleration of metabolic capacity [65].
Xenosensors, CAR and PXR

The identification of orphan receptors with unknown function would answer the question of how structurally diverse compounds were able to induce a similar pattern of xenobiotic metabolizing enzymes. The patterns of induction were generated by individual ligands binding to the same nuclear receptor; PB-like and Rif-like inducers being mediated by two independent, yet related nuclear receptors, CAR and PXR, respectively.

The constitutive androstane/active receptor (CAR, NR1I3), related to PXR in sequence and function, is the primary mediator of PB effects and necessary for induction of CYP2B isoforms [67-69]. The phenobarbitol response enhancer module (PBREM), discovered in the promoter of CYP2B genes, was used to identify the mediating nuclear receptor. Like many other orphan receptors, CAR heterodimerizes with RXRα and binds a variety of substrates, but CAR also displays a constitutive activity in cell culture models for which the inverse agonists androstanol and androstenol have been identified as repressors [70]. In its unliganded state, the folded CAR receptor positions the α-AF-2 helix into an active conformation and is able to recruit transcriptional cofactors, such as SRC-1 [70-72]. The mechanisms of nuclear translocation for CAR are poorly understood, but recently heat shock protein 90 and the cytoplasmic CAR retention protein (CCRP) were discovered to be responsible for the cytoplasmic retention of CAR in hepatocytes [73, 74]. Xenobiotic compounds such as PB and bile acids enhance the nuclear localization of CAR by a mechanism involving phosphorylation of the
receptor and not direct ligand binding [68, 75]. These CAR activators exploit the receptor’s constitutive activity to affect target gene regulation. True ligands such as androstanol/androstenol and human specific ligand CITCO (6-(4-chlorophenyl)imidazo [2,1-b]{1,3}thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime) bind CAR and mediate their respective effects by altering the tertiary receptor structure [72, 76]. CAR-mediated gene regulation results from the combined effects of CAR activators and true CAR ligands.

Despite a common profile of target gene induction, Rif-like inducers differed significantly in size, shape and structure—complicating the idea of a receptor-mediated mechanism. The orphan receptor, PXR with an exceptionally large and flexible ligand binding pocket, accommodated the variability found in this diverse group of inducers and mediated the induction of CYP3A [40, 41]. PXR is necessary and sufficient to induce CYP3A in response to xenobiotic challenge and also participates in the regulation of other CYP proteins [77-79]. Unlike CAR, PXR activity and ligand binding are simple and straightforward. PXR binds ligand, heterodimerizes with RXRα and affects target gene transcription through specific DNA response elements, but unique to PXR is the variety of ligands it recognizes and the number of target genes it regulates.

Each receptor, CAR and PXR, has an important role in xenobiotic metabolism, but it is the cumulative effect of both CAR and PXR, in a position to regulate all facets of metabolism in response to a variety of compounds that has earned them the collective moniker of ‘xenosensors.’
PXR ligands and target genes

PXR was initially defined by its ability to bind classical CYP3A4 inducers such as phenobarbital, rifampicin, dexamethasone, RU486, etc. [1, 80, 81] (Figure 2). The inductive effects of these compounds on CYP3A expression were known well before the discovery of orphan receptors and these early investigations identified necessary response elements in the proximal and distal promoter regions of CYP3A4 [82, 83]. Competition binding assays determined that PXR was able to bind DR3 and ER6 elements found within the CYP3A4 promoter [5]. The orphan receptor, PXR, recognizing a significant variety of ligands and binding specific response elements in the CYP3A4 promoter, was identified as the primary receptor mechanism responsible for mediating xenobiotic-induced effects on CYP3A4 and other metabolic target genes.

Since its discovery, many known pharmaceutical CYP3A4 inducers have been added to the list of PXR ligands. Pharmaceuticals such as chemotherapeutics [84], anti-viral protease inhibitor [85], hypolipidemic drugs [86], and antimycotics [87] are also PXR ligands. Not limited to pharmaceutical ligands, PXR binds environmental contaminants such as organochlorine pesticides [88] and phthalate esters [89]. Along with new ligands, research identified new target genes for PXR regulation—glucuronosyltransferases (UGT) 1A [90], multidrug-resistant gene 1 transporter (MDR1, also known as P-glycoprotein), and multidrug resistance-associated gene 2 transporter (MRP2) [20, 91, 92]. PXR was predominantly recognized as a receptor for exogenous compounds and mediator of xenobiotic
metabolism, until endogenous bile acids were identified as ligands for the orphan receptor. In response to lithocholic acid (LCA), PXR displayed coordinated regulation of CYP7A1 and the organic anion transporting polypeptide 2 (OATP2) transporter—decreasing conversion of cholesterol to bile acids (down-regulating 7A1) and increasing efflux of the toxic bile acid (upregulating OATP2) [93, 94]. In comparison to the global activation of metabolic target genes suggested by some PXR ligands, the regulatory effects of LCA via PXR possess the finely-tuned quality of a ligand-specific response; perhaps illustrating the difference between endogenous ligand activation of PXR and exogenous ligand activation.

Consequences of PXR activation and CYP3A4 induction

Participating in the metabolism of both endogenous and exogenous substrates implies that PXR is positioned to be a regulator or ‘xenosensor’ of overall xenobiotic exposure. However, this also implies critical endogenous pathways are susceptible to PXR dysregulation as well. While research to define new PXR target genes or identify new PXR ligands are important, these studies are unable to estimate the impact of PXR transcriptional activation on other systems or other co-administered xenobiotics. Given an environment of multiple exposures to xenobiotics (dietary intake, environmental pollutants, multi-drug therapy), the consequence of PXR activation and CYP3A4 induction are real and potentially life-threatening.
The herbal remedy, St. John’s Wort (SJW), is a commonly used antidepressant whose psychoactive component, hyperforin, is a ligand for PXR. Women taking SJW in addition to oral contraceptives discovered a decreased effectiveness of their birth control method. Hyperforin from the SJW bound PXR, increased the expression of CYP3A4, and subsequently metabolized the synthetic hormones, decreasing their effectiveness [95, 96]. This interaction of SJW and oral contraceptive co-administration illustrates the ability of PXR to regulate hormone levels. This and other examples have raised concern about the ability of PXR to cause endocrine disruption via enzyme induction. In humans, the primary enzyme responsible for testosterone metabolism is CYP3A4 and PXR ligands, such as diethylhexylphthalate (DEHP), have been demonstrated to cause testicular toxicity and testicular dysgenesis syndrome [97, 98]. Furthermore, treatment of primary human hepatocytes with DEHP displayed increased enzyme activity and increased production of the testosterone metabolite, 6-β-hydroxytestosterone (Cooper, BW, unpublished manuscript). These data suggest that PXR activation has an unexpected side effect of altering endogenous hormone levels. Recognizing that compensatory mechanisms for hormone homeostasis are many, this activation by PXR may have no effect; however, under altered circumstances of hormone synthesis or other physiological condition, this PXR-mediated change in hormone levels could lead to pathogenesis of disease.

In the clinical context, combined, or multi-drug, therapy is used to treat conditions such as cancer and HIV and in populations such as the elderly [99-102].
Many of the co-administered drugs are PXR ligands and CYP3A4 substrates creating an environment of altered pharmacokinetics that may affect the efficacy of some, all or none of the co-administered drugs [103]. With this in mind, it has been estimated that adverse drug reactions are the fourth largest killer in the western world and responsible for 100,000 deaths a year in the US. Patients given the antibiotic, rifampicin and the immunosuppressant, cyclosporin displayed decreased plasma levels of cyclosporin and a decrease therapeutic effect of drug treatment [104]. This outcome was also observed with co-administration of St. John’s Wort and cyclosporin [105] and also with co-treatment of rifampicin and the antiarrhythmic, quinidine [106]. In the case of prodrugs requiring metabolic activity to generate the biologically active molecule, co-administration of CYP inducing xenobiotics could increase the production of reactive pharmaceutical to toxic levels. Co-administration of the diabetes drug, troglitazone, which induces CYP3A activity, and acetaminophen results in the increased production of the toxic, quinone acetaminophen metabolite, NADQI and increased toxicity [107].

Adverse drug reactions by PXR activation and CYP3A4 induction are a major health concern. Interestingly, many drug-drug interactions occur where the inducer of CYP3A4 expression is not a therapeutic agent but a dietary component or herbal supplement. This underscores the importance of understanding the molecular mechanisms of drug interactions for their application to a variety of combinatorial exposures.
3. NR1I2; the Pregnane X Receptor, PXR

The discovery of orphan receptor, PXR

In 1998, a trio of publications independently identified an orphan nuclear receptor with the ability to bind well-characterized response elements in the CYP3A4 promoter that is now referred to as the pregnane X receptor. PXR, also named the steroid and xenobiotic receptor (SXR) and pregnane activated receptor (PAR), was found to be expressed primarily in metabolic tissues such as liver and small intestine and demonstrated the ability to bind steroid metabolites and known CYP3A4 inducers [1, 2, 5]. Kliewer and co workers [3] discovered the mouse PXR just months before the human PXR publications and characterized the novel signaling pathway. Derivatives of the C21 steroid, pregnane, were the first identified ligands of this orphan receptor, and thus inspired its name. At the time of this discovery, the effect of steroids on the induction of metabolizing enzymes were known but the mechanism for this action was not [108, 109]. In addition, the newly cloned receptors of steroid hormones were unable to mediate the observed effects on metabolic induction [110, 111]. Orphan receptors offered the promise of novel signaling pathways yet to be determined, as discovered with PPAR [112], FXR [113] and LXR [114, 115] and their ability to bind endogenous fatty acids, retinoids and oxysterols, respectively.

Differences in the ligand profiles of mouse PXR (mPXR) and human PXR (hPXR) were observed. Kliewer et al. [3] identified the ability of mPXR to bind the pregnane derivative, pregnenolone 16α-carbonitrile (PCN). In all studies of the
hPXR, PCN failed to bind the human receptor and activate the CYP3A promoter. Conversely, the known CYP3A inducer, rifampicin, was discovered to be a ligand for hPXR but not for mPXR [5]. Transgenic mouse models were utilized to better characterize these species-specific differences of PXR, *in vivo*. PXR-null mice confirmed the role for PXR in CYP3A induction, as they were unable to upregulate CYP3A expression in response to PCN challenge. Humanized PXR mice, expressing the human PXR transgene in place of endogenous mPXR, induced CYP3A expression in response to the human PXR-specific ligand, rifampicin and were no longer responsive to the mouse-specific ligand, PCN [77]. This observation of ligand-dependent variability in CYP3A induction across species is not novel, but the discovery of species-specific versions of PXR provides a mechanism for the species-specific induction profile. These findings explain the observed ability of CYP3A promoter regions to adopt the xenobiotic induction profile of the host cell when transiently transfected [83]. The combined impact of these observations identifies PXR as the structural determinant of CYP3A induction in response to xenobiotics.

Orthologous PXR sequences have been identified in a number of species, including pig, dog, rabbit, fish and rhesus monkey [116, 117]. Only the rhesus monkey displays significant shared identity (100% DBD, 95% LBD) with the human receptor supporting evolutionary distance as a factor in the divergence of PXR sequences across species. The rodents, mouse and rat orthologs display ~78% LBD shared identity, while the rabbit, pig and dog LBDs share 82-87% identity
when compared to human PXR. Of the receptors that have a defined DBD, the conservation of sequence is much greater, representing 94-100% shared identity [116]. These findings support the notion that orthologs of PXR across species execute the same function of upregulating metabolizing enzymes, but do so in response to a xenobiotic binding profile unique to each individual species [32, 118].

**Unique qualities of PXR**

PXR belongs to a superfamily of structurally-related proteins, yet several unique attributes distinguish PXR from other nuclear receptors. The first distinguishing characteristic of PXR to be determined was the variety of structurally-unrelated ligands it was able to bind. Ligands for PXR include: the small single-ringed hypolipidemic drug, SR12813 [41]; the macrocyclic antibiotic, rifampicin [1, 5]; the bulky chemotherapeutic, taxol [84, 119]; and cholesterol derivatives, LCA and PCN [3, 93, 120]. There seemed to be no required shape or functional group that determined PXR binding. This promiscuous ligand binding behavior is in stark contrast to the specific interaction of steroid ligands with their cognate steroid receptor. Steroid/receptor interactions supported the theory of a specific ligand binding pocket designed for a single ligand; however, discovery of synthetic compounds and phytochemicals have demonstrated an ability to activate or antagonize these highly specific receptors, contrary to the specific interaction initially characterized [121, 122]. In additional contrast with steroid receptors, PXR and other orphan nuclear receptors exhibit low-affinity ligand binding in the micro- to millimolar range [19].
Structurally, the promiscuous ligand binding of PXR is in direct relation to a ligand binding pocket (LBP) considerably larger than that of other crystallized receptors (~1100-1500 Å³) [40, 41]. On par with PXR, the LBD of orphan receptor SF-1 (NR5A1) has been resolved with a LBP volume of ~1600 Å³, while most steroid receptors have a LBP volume of ~500 Å³ and some orphan nuclear receptors lack a ligand binding pocket altogether [39, 123, 124]. Substitutions at α-helices 2 and 6 allow for the expanded size and flexibility of the PXR LBP. Within the expansive LBP, small ligand molecules, like SR12813, are able to bind in a number of orientations, making a distinct pattern of interactions with pocket residues for each orientation [40]. A scenario like this allows for varying degrees of receptor activation based on ligand orientation, but such an observation was not made in this case.

PXR also diverges from other nuclear receptors in the number and variety of target genes it regulates. As mentioned above, this orphan receptor, defined by regulating the CYP3A4 promoter, has now demonstrated the ability to regulate other CYP isoforms, phase II conjugating enzymes and transporter proteins effecting all facets of endogenous and xenobiotic metabolism [5, 79, 90, 125-129]. PXR does not accomplish all these functions at once, nor in response to any one ligand, but the total of its functional potential represents a global regulation of metabolism.

**PXR isoform variants**

Alternatively spliced receptor isoforms are found throughout the nuclear receptor superfamily. Receptors such as GR, PR, and PPARα are expressed as
isoform variants that differ by alternative splicing or alternate exon/promoter usage. It is estimated that up to 60% of all human genes have an alternate isoform, greatly increasing the protein diversity from a single gene [51, 130]. The variety of receptor isoforms allows for tissue-specific expression, altered ligand binding, or a more specialized biological role.

The initial discovery of mPXR identified two PXR isoforms, mPXR.1 and mPXR.2 [3]. Mouse PXR.2 was the result of an alternative splicing event at an internal exon:exon junction removing part of the PXR ligand binding domain. In transactivation experiments, mPXR.2 displayed a limited ligand binding profile, but demonstrated a comparable ability with mPXR.1 to bind DR3 elements in the rat CYP3A1/2 promoters in mobility shift assays. The LBD deletion causes a predictable decrease in the extent of ligand binding, but the remaining regions of the protein, i.e., DBD remain functional. The overall result is a functional receptor that responds to only a small portion of recognized ligands.

Human PXR was initially identified as a 434 amino acid (aa) protein made up of 9 exons [1, 2, 5]. Among the trio of PXR publications, only one identified an additional isoform, called human pregnane activated receptor 2 (hPAR-2) with extra 5' sequence resulting in an N-terminal addition of 39 aa [1]. Shortly after PXR characterization, a splice variant mimicking the LBD deletion seen in mPXR.2 was discovered in normal and neoplastic human breast tissue [8]. According to the NCBI sequence database, the 434 aa isoform is defined as PXR 1 (NM_003889), the 473 aa isoform with a 39 aa N-terminal addition is defined as PXR 2 (NM_022002),
and the 397 aa isoform with a deletion in the LBD is defined as PXR 3 (NM_033013). Recent publications have identified additional isoform variants produced from alternative splicing events deleting additional exon sequence or entire exons generating truncated proteins and in some cases, no protein at all as a result of a premature termination codon [7, 9]. Little is known about the function of these alternate isoforms or their contribution to the overall biology of PXR function.

The majority of recognized PXR isoforms, including PXR 3 and those discovered by Fukuen et al [9] and Lamba et al [7], represent alternative splicing events within the PXR message and could be predicted simply to limit function already attributed to the wild-type PXR 1 isoform (based on the limited function of truncated mPXR.2). The variant PXR 2 is different from all other isoform variants—PXR 2 shares all sequence (exon 2 through exon 9) with PXR 1 and has an additional 5' region adding 39 aa to the PXR 2 protein. Compared to truncated isoform variants which demonstrate a lesser receptor function, the initial characterization of PXR 2 demonstrated comparable, if not slightly greater, transactivation ability than PXR 1 [1]. Our analysis of published PXR sequences, both genomic and cDNA, determined that PXR 1 and PXR 2 had separate and distinct first exons. For PXR 1, translation initiation began in exon 2 leaving all of its first exon as a 5' untranslated region (UTR); but the distinct first exon of PXR 2 contained an additional ATG, initiating protein translation within the first exon and adding 39 N-terminal amino acids. Having alternate first exons also means that PXR 1 and PXR 2 have distinct proximal promoter regions directly 5' to those
alternate exons. This presents the possibility of altered temporal and spatial expression of these two variants [52]; with the additional consideration of a specific function that could be attributed to the N-terminal addition [131], the unique promoters and first exons of PXR 1 and PXR 2 could generate considerable variability in PXR expression. While these specifics of PXR have yet to be discovered, the concept is not without precedent within the nuclear receptor superfamily. Alternate isoforms of the progesterone receptor, PR-A and PR-B, differ by distinct first exons resulting in an N-terminal addition to variant PR-B [50]. PR-B demonstrates a greater transactivation ability, attributed to its N-terminal addition, and the distinct promoter regions for PR-A and PR-B result in significant differences in tissue-specific expression [53-55].

Regulation of PXR expression

Not surprisingly, following the discovery of a novel orphan receptor with the ability to bind an unprecedented variety of structurally-unrelated ligands and regulate numerous metabolic target genes, the majority of PXR research has focused on identifying new ligands and new target genes. However, a complete understanding of PXR biology also includes knowledge about its expression and regulation.

The first discoveries of PXR regulation were made while investigating glucocorticoid-mediated regulation of CYP3A4 induction [10]. Known to work through a non-GR mediated pathway, the ability of PXR to bind dexamethasone suggested an alternate mechanism [1, 5, 110]. Two publications confirmed that the
PXR message was upregulated by glucocorticoid treatment and that PXR was the direct mediator of glucocorticoid-induced CYP3A4 expression [10, 132]. Studies with the rat PXR demonstrated an induction of PXR message in response to PCN treatment, suggesting the ability of PXR to regulate its own expression [6]. Investigations in the developing embryo have discovered the expression of liver-enriched transcription factor HNF-4α is necessary for fetal expression of PXR [11]. The collective publications on PXR promoter regulation are few, but all share the suggested involvement of other nuclear receptors in the regulation of PXR expression. A recent publication added the discovery of a responsive element for PPARα to the PXR promoter region [133].

Common to all publications regarding PXR regulation to date is the absence of investigations concerning PXR 2. Whether using techniques that fail to differentiate between isoforms or exclusively characterizing the PXR 1 promoter region, all publications have failed to include the lesser-recognized isoform PXR 2. With a proximal promoter limited by the genomic placement of the first exon of PXR 1, PXR 2 regulation may be restricted to a finite region, but the possibilities of regulating PXR 2 expression have yet to be explored.

4. Hypothesis and Rationale

The critical position of PXR in the regulation of xenobiotic metabolism demands a greater understanding of its isoform variants, their individual regulation and their contribution to the collective PXR function. Human PXR 2, when first
discovered, was thought to be simply a splice variant of the wild-type human PXR 1 with little individual character, much like other alternate isoform variants found in mouse and rat. In fact, human PXR 2 differs from PXR 1 by a unique first exon and a distinct promoter region which give PXR 2 additional N-terminal sequence and the potential for autonomous regulation, respectively. Despite the lack of research on PXR 2, there is precedent for the significant contribution of alternate receptor isoforms within the nuclear receptor superfamily.

Distinct promoter regions are most significant among the differences between PXR 1 and PXR 2 by offering the greatest potential for altering receptor isoform function through individual temporal and spatial regulation. The dexamethasone-mediated induction of PXR, mentioned above, was determined by measuring mRNA expression and was unable to distinguish the induction by individual isoform. Taken together, the observed regulation of PXR by dexamethasone and the presence of two distinct promoters allow us to explore the differential regulation of receptor isoforms and investigate the importance of PXR 2. Specifically, we sought to identify glucocorticoid response elements (GREs) in the promoters of PXR 1 and PXR 2 and further understand the independent regulation of each isoform. In the broad sense, we wanted to further investigate the consequences of alternate receptor isoforms and characterize the functions and contributions of PXR 2 to the overall PXR receptor biology.
In the following manuscript, we:

1) illustrated the functional significance of PXR 2 with promoter activation studies.

2) measured the relative expression of total PXR and PXR 2 in human tissue.

3) experimentally identified transcription start sites for PXR 1 and PXR 2, thus delineating proximal promoter regions for further analysis.
Figure 1. Common structural domains of nuclear receptors. Each domain has independent and overlapping functions that contribute to overall receptor function.

A/B – N-terminal domain, AF-1; C – DNA-binding domain; D – hinge domain; E – ligand-binding domain, AF-2; F – C-terminal
Figure 2. Structural variety of PXR ligands.
Figure 3. Diagram of human PXR isoforms, PXR 1, PXR 2 & PXR 3. Based on the NCBI sequences, the isoforms drawn above illustrate the N-terminal addition to PXR 2 and the LBD-deletion of PXR 3.
5. References


1. 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'UTR and TSS characterizations of the human PXR gene, and subsequently endeavored to define TSSs and proximal promoters for isoforms PXR 1 and PXR 2. RT-PCR 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 nuclear 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 while modest 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.

2. 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) (Kliewer et al., 1998; Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann 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 RXRα (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), phthalate esters (Takeshita et al., 2001), brominated 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 downstream
target genes. Endogenous compounds such as pregnane 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 (Gerbal-Chaloin et al., 2001; Goodwin et al., 2001; Lehmann et al., 1998); 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 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 CYP3A4 xenobiotic inducers was the synthetic glucocorticoid dexamethasone whose signaling was not mediated directly by the glucocorticoid receptor (GR; NR3C1). Pascussi et al. (2001) demonstrated 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 (farnasoid X receptor (FXR; NR1H4)
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. To date, PXR mRNA expression has also been reported in the stomach, heart, brain, breast tissue and testes. In each of these tissues, the abundance of PXR, 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, 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 need for better understanding of PXR regulation, isoform expression and the cumulative effect on the regulation of xenobiotic metabolism.

3. 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 6-8h 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, 10pmol of each primer (see Table 1) was end-labeled with $\gamma^{32}$P-ATP (sp. act. 3000Ci/mmol) using T4 polynucleotide kinase according to manufacturer’s instructions (Promega, Madison WI). After quenching with 0.5M EDTA, 20ug total RNA from human liver was added and the sample was precipitated with 3M sodium acetate (pH 5.2) and 2.5 vols of 100% ethanol at -20°C for 30m to overnight. To anneal, the RNA and primer were resuspended in dH2O and incubated at 70°C for 10m 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 (0.5mM each dNTP) and 15U RNasin (Ribonuclease Inhibitor, Promega, Madison, WI). The reaction was incubated at 42°C for 30m and quenched with 1 vol 2X loading dye
(95% formamide; 18mM EDTA; 0.025% SDS, xylene cyanol, and bromophenol blue). Each sample was heated to 90°C for 10m before loading on sequencing gel.

A 2.5kb 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 2.5kb 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 (8M 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 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 x 10^5 cells per well. Twenty-four hours later, cells were transfected with 1.0ug CYP3A4-luc and 0.1ug 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 48hrs with vehicle control, DMSO and 10uM rifampicin (Sigma-Aldrich, St. Louis, MO). Following treatment, cells were lysed and 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 is 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.
4. 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 45kb 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 25nt 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 ~110bp region and a ~160bp 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 termination product distinct from that of primer PXR1A stopping at ‘GC’ residues in its ladder. Despite also stopping at a ‘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.

Primer extension analysis of the PXR 2 transcript confirmed a TSS in the ~160bp region between primer PXR2.3 and PXR2.4. Primer PXR2A generated two termination products with ‘C’ and ‘T’ start sites separated by 3nt. The upper ‘C’ termination band appears to be more abundant; the lower ‘T’ termination band may be a product of stuttering in a GC-rich region or a simple wobble at the start of transcription. The primer PXR2B extension reaction also generated two termination products, but with significant distance between them. 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 in the vicinity of the ‘e*’ start site discovered by Kurose et al. (2005) (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 the 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).

**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 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 prototypical human ligand, rifampicin, cells transfected with PXR 1 increased CYP3A4 promoter activity 17.6-fold over vehicle treated control. PXR 2 transfected cells also responded to rifampicin treatment with a 16.7-fold increase in CYP3A4 promoter activity. In this experiment, PXR 1 and PXR 2 illustrated comparable ability to activate transcription via interactions with the PXR-responsive CYP3A4 promoter.

**5. 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 (aa) was discovered in tandem with PXR 1 [Bertilsson, 1998 #44], 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, like PXR 2 and PXR 3, 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; Mulac-Jericevic et al., 2003; Richer et al., 2002). Here, 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 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).

The RT-PCR results mapping the 5’ end of PXR 1 differed from the start sites suggested by Zhang et al. (2001) and the NCBI reference sequence NM_003889 (mentioned above). Kurose et al. (2005) published four TSSs for PXR 1 identified by 5’RACE, and our RT-PCR data confirmed only one. 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^A at nt45535) and one further downstream (+1^B 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). However, primer extension analysis, as a more precise technique for 5’ exploration serves to refine the 5’RACE conclusions. Furthermore, despite the use of multiple primer extension primers, we failed to detect a termination product in the region of the ‘c*’ start site, suggested as the major PXR TSS by Kurose et al. (2005).
The mapping of the 5’ end of PXR 2 also began with differing results from the start sites proposed by Zhang et al. (2001) and its 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 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 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 serve as a minor start site for transcription.

Our new TSSs add as much as 300bp to the 5’UTR of PXR 1 and 120bp 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 for PXR 1 and PXR 2, 600bp of proximal promoter 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). Response elements were found for: liver-enriched factors such as HNF1 and HNF4, C/EBP family members, and other nuclear receptors such as PPAR and GR in the proximal promoters of both PXR 1 and PXR 2. Additionally, binding sites for the transcriptional repressor, NF-κB 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 promoter studies are confounded by the use of different TSS and promoter region. 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.
Table 1. All oligonucleotide primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ → 3’)</th>
<th>Position(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PXR1.1</td>
<td>AGGACGGGAAGAGGAAGCA</td>
<td>45895</td>
</tr>
<tr>
<td>PXR1.2</td>
<td>GGTAAAGGACAGAGACCCTCA</td>
<td>45598</td>
</tr>
<tr>
<td>PXR1.3</td>
<td>CACTATCCAGGGAGGTGGTTTC</td>
<td>45524</td>
</tr>
<tr>
<td>PXR1.4</td>
<td>CATCTCACACCCTTTCCCTCTC</td>
<td>45415</td>
</tr>
<tr>
<td>PXR2.1</td>
<td>TTTGGCCTGCTGGGTAG</td>
<td>46524</td>
</tr>
<tr>
<td>PXR2.2</td>
<td>CCTGCACCTAGCTCTAGAAAAA</td>
<td>46338</td>
</tr>
<tr>
<td>PXR2.3</td>
<td>GTCTCCTCATTTCCTTGTGGGAG</td>
<td>46257</td>
</tr>
<tr>
<td>PXR2.4</td>
<td>GGGAGAAGTCCGAGCAAAGA</td>
<td>46082</td>
</tr>
<tr>
<td>PXR RP</td>
<td>GAAGTCGGAGGTCCCCCAATCT</td>
<td>70510</td>
</tr>
<tr>
<td>PXR1A</td>
<td>AGGAAAAGGGGAAGAGAGGAGG</td>
<td>45686</td>
</tr>
<tr>
<td>PXR1B</td>
<td>TGCTTTTTTCAGGACAGGAG</td>
<td>46015</td>
</tr>
<tr>
<td>PXR2A</td>
<td>TTTTTCTAGGAGGCTAGTGCAGG</td>
<td>46360</td>
</tr>
<tr>
<td>PXR2B</td>
<td>CTTGAGGTGTTATTTCCTG</td>
<td>46470</td>
</tr>
</tbody>
</table>

\(^a\)Position numbers based on genomic sequence AF364606.
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 20µg of total RNA and 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 48hr with DMSO vehicle control or 10uM rifampicin. Data is expressed as mean fold induction ± SE. Statistical significance was determined by ANOVA, Dunnett’s mean comparison test when compared to ‘pcPXR1’ vehicle control, ****p<0.0001. 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 PXR 1 5'UTR by ~300bp (+1A) or 60bp (+1B) and added ~120bp (+1B) to the 5'UTR of PXR 2. The alternate TSS for PXR 2 (+1A) is shown but considered to be a minor start site. Three receptor isoforms produced from our TSSs are shown below.
REFERENCES


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. \textit{Mol Pharmacol} \textit{54}: 1113-1117.


The objective of this research was to present a larger picture of PXR biology and function through proven molecular techniques and a simple strategic approach. The importance of PXR in regulating xenobiotic metabolism in response to a variety of ligands from endogenous metabolic byproducts and exogenous pharmaceuticals has been widely recognized, but the lack of research on topics such as promoter regulation and alternate receptor isoforms seems counterintuitive to such a critical nuclear receptor pathway. It was our initial goal to help fill that void, but our investigation into the regulation of the PXR promoter(s) discovered contradictory sequence data and inaccurate or inconsistent assumptions made by other published investigators. Before proceeding with the PXR promoter studies, it became our task to characterize the 5’ region of the PXR gene in a scientifically sound way in order to eliminate the contradictions and inconsistencies.

The existing TSS data consisted of the sequence published by Lehmann et al. (AF061056) [1] as discovered in a cDNA library screening and a recent publication using 5’RACE (Rapid amplification of cDNA ends) to characterize the 5’ region of the PXR gene [2]. The Lehmann published sequence serves as the unofficial reference for transcription and translation start site information based on its reference in other sequences and publications; yet none of the experiments were specifically designed to generate such data nor was the data confirmed by other techniques. Recently, 5’RACE analysis of the 5’ region of PXR confirmed a major
TSS for PXR 1 ~30nt downstream from the site identified by Lehmann et al. [1] while also identifying three other minor start sites for PXR 1 and a TSS for PXR 2 [2]. This publication represents the first characterization of PXR 1 and PXR 2 since the cDNA library screenings, which identified each receptor isoform; yet again, these 5'RACE findings weren’t confirmed or validated by any other molecular technique. The use of 5'RACE, while powerful under the right circumstances, can be a highly error-prone technique requiring the sequencing of hundreds of clones to identify your target. Contributing to the variability, cDNA libraries, which were utilized as template for the 5'RACE experiments, have their own limitations based on the quality of library construction and commonly have sequencing or cloning errors that compromise the 5’ ends of a cDNA transcript [3, 4]. In addition to the publications above, a genomic sequence for PXR (AF364606) [5] and a cDNA reference sequence for each of the PXR isoforms 1(NM_003889), 2 (NM_022002), & 3 (NM_033013) were available from the NCBI database representing the sum total of information on the sequence and TSS of PXR.

Utilizing the published genomic PXR sequence, we chose to design experiments using sequence-specific primers for RT-PCR and primer extension analysis to explore the 5’ region of PXR 1 and PXR 2, instead of a technique like 5'RACE. Our preliminary RT-PCR results for both PXR 1 and PXR 2 differed from all recognized TSSs. On its own, these data only served to further complicate the identification of a PXR TSS, so primer extension analysis was added to confirm our RT-PCR conclusions. The primer extension data did confirm our RT-PCR results,
but also identified sites that the RT-PCR experiments would be expected to miss. This is precisely why concerns were raised over the conclusions drawn by Lehmann et al., Kurose et al., and other investigators referencing their work(s) from unconfirmed data [1, 2, 5-7]. Our TSS results, derived directly from RNA template and confirmed by two molecular techniques, represent experimentally sound conclusions based on a coordinated experimental approach.

Despite the inconsistencies we observed in the TSS identification for PXR, a number of investigators proceeded with promoter evaluation studies. Collectively, they identified response elements for liver enriched HNF-3 and HNF-4, co-activators Oct-1 and C/EBP family members, and other nuclear receptors PR, GR, PPAR\(\alpha\), and VDR [2, 6, 7]. While each publication denoted the TSS and promoter region used, our data suggests all other TSS assumptions to be inaccurate and changes the interpretation of the promoter studies. Defined by prediction matrices, the putative response elements still exist in the promoter sequence but the genomic context of those elements may have changed.

Contrary to the promoter evaluation studies, Pascussi et al. presented PXR regulation data not affected by new TSS results [8, 9]. Discussed earlier, research into the well-documented glucocorticoid-mediated regulation of CYP3A4 expression discovered PXR to be the receptor interacting directly with the CYP3A4 promoter, while the direct effects of GR were upregulating PXR expression. These observations were made by measuring the RNA message via RNase protection assay (RPA) and not through any promoter related analysis. The RPA data
demonstrated a dose- and time-dependent increase in PXR message in response to glucocorticoid treatment that followed the induction of known GR-mediated genes such tyrosine aminotransferase (TAT). These data clearly illustrated the regulation of PXR by glucocorticoids mediated through GR and suggested the presence of a glucocorticoid response element (GRE) in the PXR promoter. These data, put in the context of two receptor isoforms, PXR 1 and PXR 2 that differ in their proximal promoter regions, present the possibility that GR may regulate the expression of one or both isoforms. However, use of a RPA probe that recognized a region of PXR message common to all isoforms prevents the ability to evaluate this regulation data based on individual isoforms. Understanding this was not the author’s objective, we chose this data as a foundation for investigating the unique promoter regions of PXR 1 and PXR 2.

In addition to the TSS data for PXR 1 and PXR 2, we present data supporting the inclusion of PXR 2 when investigating the regulation of PXR expression. Based on our small human cross-section, PXR 2 is expressed at relatively low levels, contributing only a small portion to the total PXR expression measured from each individual. Despite the significant variability in total PXR expression across individuals, the expression of PXR 2 remained at a relatively consistent percentage. One explanation would identify PXR 2 as a constitutively expressed isoform with lower abundance yet consistent expression, while PXR 1 is the inducible isoform whose expression would vary widely based on exposure to inducing compounds. Discovering comparable transcriptional activity of PXR 1 and PXR 2 reinforces the
importance of individual isoform regulation suggesting each isoform may be required to fulfill the same function, but under different circumstances or cellular conditions. Evidence exists that alternate isoforms of other nuclear receptors, such as PR and GR, demonstrate significantly divergent activities that contribute to the collective function of receptor expression [10, 11]. Isoforms PR-B, GR 1A3, and GRβ represent altered receptor function as a product of N-terminal addition, alternate first exon and promoter region, and alternative splicing, respectively [12-14]. The alternate properties of PXR 2, a distinct promoter region and N-terminal addition, can reasonably be expected to alter receptor isoform function. Altogether, PXR 2 demonstrates unique attributes that under a variety of circumstances could alter the collective biology and function of PXR expression.

References


Mechanisms of Cytochrome P450 Induction

Leslie M. Tompkins and Andrew D. Wallace *

* Department of Environmental & Molecular Toxicology, Campus Box 7633, North Carolina State University, Raleigh, North Carolina 27695-7633, USA.

# To whom correspondence should be addressed. Tel.: 919-515-8520; Fax: 919-515-7169; E-mail: andrew_wallace@ncsu.edu

Abstract

Cytochrome P450s (CYPs) are important heme containing proteins that play important roles in the metabolism of xenobiotics and endogenous compounds. The oxidative metabolism of drugs, environmental chemicals, hormones, and fatty acids by CYP enzymes are critical pathways aiding in their excretion from the body, but in some cases metabolism may lead to bioactivation and enhanced toxicity. The expression and activity levels of CYPs can be elevated by a process of induction involving the activation of key transcription factors. The mechanisms by which CYP3A4, 2B6, and 1A1 are induced involving the activation of the transcription factors pregnane X receptor (PXR), constitutive androstane receptor (CAR), and aryl hydrocarbon receptor (AhR) will be discussed.

Keywords: Cytochrome P450 induction, pregnane X receptor, constitutive androstane receptor, aryl hydrocarbon receptor

Introduction

The induction of cytochrome P450 (CYP) enzymes by xenobiotics is a major concern due to the enhanced metabolism of pharmaceutical drugs and endogenous substrates. The oxidative metabolism of drugs to more polar metabolites is an important mechanism allowing for the elimination of xenobiotics. Over the last ten years, major advances have been made
in the understanding of the receptors and mechanisms involved in the induction of CYP enzymes, such as CYP2B6 and CYP3A4. With the exposure to multiple drugs or environmental chemicals, the potential exists for CYP induction to reduce the circulating drug concentrations or endogenous hormone levels. In the exposed patient, this can lead an ineffective therapy. Alternatively, the increased metabolism of certain CYP substrates may lead to bioactivation and the formation of reactive metabolites causing enhanced toxicity.

CYP Induction

The mechanisms by which CYPs are induced has been an area of extensive research for many years. Many of the CYPs are induced in humans including CYP1A, CYP2A, CYP2B, CYP2C, CYP2E1, and CYP3A by a diverse array of compounds including drugs, industrial chemicals, natural products, and ethanol. The inducible CYPs make up a large percentage of the CYPs in the human liver and are responsible for the metabolism of a large proportion of pharmaceutical drugs. In most cases, induction of CYPs occurs by a process involving de novo RNA and protein synthesis that has been demonstrated in studies using transcription and translation inhibitors (1). The induction of many CYPs occurs by a similar mechanism where ligand activation of key receptor transcription factors including pregnane X receptor (PXR), constitutive androstane receptor (CAR), aryl hydrocarbon receptor (AhR) and others, leads to increased transcription (Table1). The induction of CYPs is highly conserved and is found not only in humans, but also many other species including rodent models. An alternative mechanism of CYP induction involves compounds that stabilize translation or inhibit the protein degradation pathway. For example, ethanol can induce CYP2E1 by such mechanisms leading to enhanced formation of reactive acetaminophen metabolites and enhanced hepatotoxicity (2).

PXR and Induction of CYP3A

In the human liver, CYP3A4 is the most highly expressed CYP enzyme and is highly inducible by a wide variety of xenobiotics. The mechanisms by which structurally diverse xenobiotics induce CYP3A4 protein expression have been extensively investigated. Induction of CYP3A4 can also have serious toxicological consequences as a result of
increased drug metabolism that contributes to drug-drug interactions, the bioactivation of xenobiotics to carcinogenic or toxic metabolites, and possibly endocrine disruption. For example, co-administration of the CYP3A4-inducing St. John’s Wort together with cyclosporine leads to enhanced metabolism and clearance of cyclosporine and insufficient plasma levels. Similarly, treatment of patients with the prototypical CYP3A4 inducer rifampicin, can lead to reduced plasma levels of the antidiabetic drug pioglitazone (3). Up-regulation of CYP3A4 can also exaggerate toxic responses to drugs as with rifampicin and acetaminophen co-administration (4).

In 1998, an orphan receptor was identified that bound a wide variety of steroids, steroid metabolites, many pharmacologically important drugs, and a number of xenobiotics, unlike any other nuclear receptor (5-7). The CYP3A4 induction by xenobiotics is now thought to be largely due to xenobiotic binding and activation of this orphan receptor. The Evans group named this protein the steroid and xenobiotic receptor (SXR) for its ability to bind such a wide variety of steroids and xenobiotics. The GlaxoSmithKline group named the mouse protein the pregnane X receptor (PXR, NR1I2) for its ability to strongly bind and be activated by the steroid metabolite, pregnane.

PXR is a member of the nuclear receptor superfamily and contains modular functional domains, including a DNA-binding domain and a ligand-binding domain. PXR is most closely related to the vitamin D receptor (VDR) and, like VDR, binds to DNA elements as a heterodimer with retinoid X receptor alpha (RXRα). PXR response elements have been well characterized, with the receptor binding to direct repeat with a 3 nucleotide (nt) spacer (DR3), everted repeat with a 6nt spacer (ER6), and a direct repeat with a 4nt spacer (DR4). These elements have been identified in the human CYP3A4 and rat CYP3A23 promoters and sites are bound by PXR in the presence of xenobiotics. X-ray crystallography of the ligand binding domain of PXR determined that PXR has a much larger ligand binding pocket when compared to other receptors; enabling PXR to bind such a wide variety of ligands (8). Since the initial identification of PXR, a growing list of compounds have been found to bind and activate this receptor including the chemotherapeutic agents, environmental contaminants, and antibiotics (9-12).

The promiscuous nature of PXR binding to so many xenobiotics led Blumberg and Evans to propose PXR acts as a nonspecific xenobiotic-sensor (7) (Figure 1). This hypothesis has been supported by the linkage of PXR to the regulation of an increasing
number of proteins important in detoxification pathways. PXR target genes include not only members of the CYP3A, 2B, and 2C families, but other metabolic proteins such as glutathione S-transferase, multi-drug resistance gene that encodes P-glycoprotein (ABCB1), multidrug resistance-associated proteins (Mrps), and organic anion-transporting polypeptide 2(oatp2) (13-17). Further evidence in support of this hypothesis was established by the targeted disruption of the mouse PXR gene locus, in which PXR knockout mice failed to display CYP3A induction by xenobiotic challenge and were susceptible to liver damage when administered bile acids (18, 19). The CYP3A magnitude of induction also varies greatly between individuals in human studies and it has been suggested that polymorphisms in PXR may be important in this wide variability (20-23).

CAR and Induction of CYP2B6

It had been known for many years that key CYP isoforms were induced by phenobarbital (PB), specifically members of the CYP2B subfamily of enzymes in human, mouse, and rat, but the mechanism for this induction was unclear. In humans, CYP2B6 is responsible for the metabolism of large number of drugs including chemotherapeutics, opioids, and the HIV-1 reverse transcriptase inhibitor efavirenz (24-26). CYP2B6 is highly inducible not only by PB, but like CYP3A4 is also induced by a large number of compounds with diverse structures (27).

Studies of CYP2B promoters in rat and mouse identified critical elements necessary for PB induction which were termed a phenobarbital-responsive enhancer modules (PBREMs) (28-30). Use of the PBREM DNA element in affinity chromatography studies led to the identification of a liver specific transcription factor that bound to this element (29). This transcription factor was named the constitutive androstane receptor or constitutively active receptor (CAR, NR1I3) for its ability to interact with androstanol and adrostenol and its in vitro ability to be constitutively active. In vivo, CAR is not constitutively active, but translocates from the cytoplasm to the nucleus following activation by PB. CAR is a member of the orphan receptor subfamily and as such contains modular functional domains, including a DNA-binding domain and a ligand-binding domain. Like PXR, CAR binds to DNA elements as a heterodimer with RXRα. CAR DNA binding sites have been well characterized; with the receptor binding to a direct repeat with a 4 nt spacer (DR4). These
elements are located in the human CYP2B6 and mouse Cyp2b10 promoters and sites are bound by CAR in the presence of PB and xenobiotics. In X-ray crystallography studies, CAR was shown to have much smaller ligand binding domain than PXR. Additionally, unique structural conformations were identified that may explain CAR’s ligand independent activities (31, 32). Since the initial identification of CAR, a number of compounds have been found to activate this receptor, but whether these compounds directly bind to CAR is still controversial (27, 33). Prototypical human and mouse CAR binding compounds include 2,3,3,4,5,6-hexachlorobiphenyl, 6-(4-chlorophenyl) imidazo [2,1-b][1,3] thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl) oxime (CITCO) and 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) which have been extensively used in studies of CAR function.

The induction of CYP2B family members by PB via CAR activation is just one the groups of metabolizing enzymes regulated by this mechanism. Along with CYP2B induction, upregulation of other genes including CYP2C, CYP3A, sulfotransferases, glucuronosyltransferases, glutathione S-transferases, and transporters occurs with CAR activation (34, 35). Further evidence in support of CAR’s critical role in the CYP2B induction was established by the targeted disruption of the mouse CAR gene locus. CAR knockout (CARKO) mice failed to display Cyp2b10 induction in response to PB treatment (36). The chronic activation of CAR by PB has also been linked to the development of liver tumors in mice, which were absent in CARKO animals, but it is not yet clear if these findings are relevant to human CAR activation or human PB exposure (37).

AhR and Induction of CYP1A

In humans, both CYP1A1 and CYP1A2 are inducible by a diverse array of drugs, dietary agents, and environments contaminants. The induction of CYP1A has been intensely studied and was discovered as a result the observation that halogenated aromatic hydrocarbon (HAHs) and polycyclic aromatic hydrocarbons (PAHs) enhanced their own metabolism. Other substrates of CYP1A1 include some clinical drugs, caffeine, and eicosanoids (38). The environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was found to be a prototypical CYP1A inducer and has been widely used to study the mechanisms of its induction. CYP1A induction has been shown to be important in the metabolism of PAHs and the production of reactive genotoxic metabolites important in
cancer formation (39, 40). Similarly, induction of CYP1A by cigarette smoke is associated with toxicity and carcinogenesis.

The use of photoaffinity labeled TCDD lead to the cloning of the aryl hydrocarbon receptor (AhR) (41, 42). The AhR dimerization partner AhR nuclear translocator (Arnt) was identified after a number of mouse hepatoma cell lines were found to be non-responsive to TCDD exposure (43). AhR and Arnt belong to a family of transcription factors that contain basic-helix-loop-helix (bHLH) and Per-Arnt-Sim (PAS) domains. In the presence of ligands, the AhR-Arnt heterodimer forms and binds to xenobiotic response elements such as ‘TNGCGTG’ in the proximal and distal promoter regions of the CYP1A gene (44, 45). AhR binds not only TCDD, but a variety of ligands such as the gastroesophageal reflux drug omeprazole, naturally occurring dietary chemicals like β-naptoflavone, benzo [a] pyrene, and many others (46, 47).

CYP1A induction by TCDD and structurally diverse synthetic and environmental chemicals is an example of just one gene regulated by AhR activation. The AhR signaling pathways induce CYP1A and growing number of other genes including the metabolizing enzymes UDP-glucuronosyltransferase 1A (UGT1A1) and glutathione S-transferase A2 (gsta2) (48, 49). The dependence of CYP1A induction on AhR has been demonstrated with the targeted disruption of the mouse AhR gene locus. AhR deficient or knockout mice failed to display CYP1A induction by prototypical AhR agonist challenge (50, 51). In humans, as has been observed in different mouse strains and rodent cell lines, the wide variability in the magnitude of CYP1A induction may be due to polymorphisms of AhR and Arnt (52).

CYP Induction and Other Receptors

It is beyond the scope this review to discuss the induction of all CYP isoforms individually and the unique induction mechanisms and receptors involved in detail, but it is worth mentioning other important inducible CYPs, namely CYP4A, CYP7A1, and CYP2C. The mechanisms of CYP enzyme induction not only involves the AhR, PXR, and CAR receptors, but a number of other transcription factors including peroxisome proliferator-activated receptor (PPAR), farnesoid X receptor (FXR), liver X receptor (LXR), hepatic nuclear factor (HNF) family members, glucocorticoid receptor (GR), and CCAAT/enhancer-binding proteins (C/EBPs). Many of these transcription factors also play roles in the
previously discussed inductions of CYP1A, CYP2B, and CYP3A family of enzymes, as there is significant overlap of regulatory pathways and multiple DNA binding elements in each CYP promoter. CYP4A induction is dependent on PPAR activation by a diverse range of drugs and environmental contaminants (53). CYP7A1 regulation involves the FXR and LXR transcription factors (54).

Non-receptor Mediated Induction of CYP2E1

In the human liver, CYP2E1 is highly expressed and is inducible by ethanol, acetone, and the drug isoniazid. The induction of CYP2E1, unlike that of other CYP isoforms discussed above, involves post-transcriptional stabilization of CYP2E1 (55, 56). Induction of CYP2E1 has been shown to be responsible for bioactivation of a number of substrates to reactive metabolites, causing oxidative stress and hepatotoxicity. CYP2E1 metabolizes organic solvents, nitrosamines, and drugs such as acetaminophen. Alcohol induction of CYP2E1 alters acetaminophen metabolism leading to increase formation of the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) (57). The NAPQI metabolite is highly reactive and damages DNA, proteins, and lipid membranes. The mechanism of CYP2E1 induction involves both enhanced translation of CYP2E1 mRNA and inhibition of the proteasome ubiquitin degradation pathway (2, 58-60). The importance of CYP2E1 in acetaminophen metabolism and toxicity has been demonstrated in CYP2E knockout mice, as these mice are resistant to acetaminophen toxicity.

Methods to Study CYP Induction

Many in vitro and in vivo methods exist to study induction of CYP enzymes. It is important to investigate if pharmaceuticals or environmental chemicals have the ability to induce CYP expression that could lead to decreased drug effectiveness, drug-drug interactions, or potentially greater toxicity. With the exposure to multiple chemicals, the potential greatly increases for CYP induction and subsequent unwanted side effects. For example, the induction of CYP3A4 is important as CYP3A4 accounts for metabolism of a large proportion of drugs and endogenous steroids.
The ability of compounds to induce CYPs can be assessed in tissue culture models by transient transfections using immortalized cells expressing relevant receptors and CYP promoter-reporter plasmids. Alternatively, primary cultures of rodent and human hepatocytes have been extensively used to determine if compounds can induce CYP mRNA, protein, and metabolic activity (61-63). The use of human hepatocytes is considered the \textit{in vitro} “gold standard” to assess if a compound induces CYP isoform expression (52). Human hepatocytes have been used not only to determine if a compound induces important CYPs such as CYP3A4, but also to further investigate the mechanism(s) of induction.

The use of \textit{in vivo} models to study CYP induction are very valuable, but sometimes in animal models, such as rodents, species specific differences do not allow for accurate assessment of the potential for a compound to cause CYP induction in humans. For example, the antibiotic rifampicin is a potent CYP3A4 inducer in humans, but not in rodents. In efforts to improve mouse models of CYP induction, a number of transgenic mice have been made. Humanized transgenic mice, where the mouse gene has been disrupted and the human CYP gene has been introduced, express only human CYPs and have been very useful in the studies of human CYP1A, CYP3A4 and CYP2E1 induction, metabolism, and toxicity (47). Our recent understanding of the receptors critical in the induction of CYPs has also lead to the development of many new transgenic mouse models in which the mouse receptor gene has been replaced with the human receptor. These transgenic models include humanized AhR, PXR, CAR, and PPAR\textsubscript{\alpha} mice, which have been very useful for the study of the mechanisms of induction. For example, in humanized PXR mice, CYP3A induction is observed by the antibiotic rifampicin (19). Many other CYP and receptor knockout or humanized transgenic mice have been made which will provide better models for the study of CYP induction and its consequences.

Summary

The induction of CYPs has been studied for decades and remains a major area of active research, as much is still not known about the molecular mechanisms of induction. As new drugs and environmental chemicals are developed, the potential exists for these new compounds to cause CYP induction and unwanted side effects. In recent years, our understanding of CYP induction has lead to the development of new \textit{in vitro} and \textit{in vivo}
models, which will greatly aid our ability to assess these new compounds. Our ability to predict if compounds will cause CYP induction is still compromised by the complexity of signaling pathways involved including overlap of receptors, genetic polymorphisms, and environmental factors that influence these pathways.
Table 1. Inducible CYP isoforms

<table>
<thead>
<tr>
<th>CYP</th>
<th>Receptors</th>
<th>Prototypical Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A</td>
<td>AhR</td>
<td>TCDD</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>hCAR</td>
<td>CITCO</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>hPXR</td>
<td>Rifampicin</td>
</tr>
<tr>
<td>CYP4A</td>
<td>hPPAR</td>
<td>Thiazolidinediones</td>
</tr>
<tr>
<td>CYP7A</td>
<td>FXR/LXR</td>
<td>Bile acids/Oxysterols</td>
</tr>
</tbody>
</table>
Figure 1. PXR Mechanism of Action
References

1. **Daujat M, Clair P, Astier C, et al.** 1991 Induction, regulation and messenger half-life of cytochromes P450 1A1, 1A2 and IIIA6 in primary cultures of rabbit hepatocytes. CYP 1A1, 1A2 and 3A6 chromosome location in the rabbit and evidence that post-transcriptional control of gene IA2 does not involve mRNA stabilization. Eur J Biochem 200:501-10


11. **Schuetz EG, Brimer C, 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 54:1113-7


38. **Nebert DW, Russell DW** 2002 Clinical importance of the cytochromes P450. Lancet 360:1155-62


45. **Kawajiri K, Fujii-Kuriyama Y** 2007 Cytochrome P450 gene regulation and physiological functions mediated by the aryl hydrocarbon receptor. Arch Biochem Biophys

46. **Denison MS, Nagy SR** 2003 Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annu Rev Pharmacol Toxicol 43:309-34


49. **Dere E, Boverhof DR, Burgoon LD, Zacharewski TR** 2006 In vivo-in vitro toxicogenomic comparison of TCDD-elicited gene expression in Hepa1c1c7 mouse hepatoma cells and C57BL/6 hepatic tissue. BMC Genomics 7:80


54. **Kalaany NY, Mangelsdorf DJ** 2006 LXRS and FXR: the yin and yang of cholesterol and fat metabolism. Annu Rev Physiol 68:159-91


57. **Hinson JA, Reid AB, McCullough SS, James LP** 2004 Acetaminophen-induced hepatotoxicity: role of metabolic activation, reactive oxygen/nitrogen species, and mitochondrial permeability transition. Drug Metab Rev 36:805-22


