

UNIVERSITY OF CALIFORNIA, SAN DIEGO

Nuclear Receptor Regulation of the UDP-Glucuronosyltransferase 1 Locus
and the Role of the Pregnane X Receptor as a Tissue-Specific Master Regulator

A Dissertation submitted in partial satisfaction of the
Requirements for the degree of Doctor of Philosophy

in

Biomedical Sciences

by

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The Dissertation of Erin Janine Sinnokrak is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2010

DEDICATION

To my parents, Larry and Niki Brace,
and my husband Nicholas Sinnokrak

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LIST OF ABBREVIATIONS

AhR	aryl hydrocarbon receptor
Anxa2	annexin a2
Arnt	aryl hydrocarbon nuclear translocator
ARP1	actin-related protein of the dynactin complex 1
ATCC	American type culture collection
B[a]P	benzo[<i>a</i>]pyrene
BA	bile acids
CAR	constitutive androstane receptor
CITCO	6-(4-chlorophenyl)imidazo[2,1- <i>b</i>][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime
CN-I,-II	Crigler-Najjar Syndrome, Type I or II
COUP-TF	chicken ovalbumin upstream promoter-transcription factor
CPH	carboxypeptidase H
$C_t / \Delta C_t$	comparative threshold cycle value
CYP(s)	cytochrome(s) P450
DBD	DNA-binding domain
DMSO	dimethyl sulfoxide
DRE	dioxin response element
DRx	direct repeat, x-nucleotide spacer
E ₁	estrone
E ₂	estradiol
EDTA	ethylenediaminetetraacetic acid

EGTA	ethylene glycol tetraacetic acid
ER	estrogen receptor
ERx	everted repeat, x-nucleotide spacer
FXR	farnesoid x receptor
GA	glucuronic acid
GR	glucocorticoid receptor
Gsta 2/4	glutathione s-transferase 2/4
H ₂ O ₂	hydrogen peroxide
HAH	halogenated aromatic hydrocarbons
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HNF	hepatocyte nuclear factor
i.p.	intraperitoneal
IRx	inverted repeat, x-nucleotide spacer
LBD	ligand binding domain
LCA	lithicholic acid
LXR	liver x receptor
Ly6d	lymphocyte antigen 6 complex, locus D
MOPS	3-(N-morpholino)propanesulfonic acid
Mrp2	multidrug resistance associated protein 2
N-Cor	nuclear receptor corepressor
NR(s)	nuclear receptor(s)
NR1I2	nuclear receptor family 1, subfamily I, group 2
NTCP	Na(+)/taurocholate transport protein

Oatp4	organic anion transporter protein 4
OCS	oral contraceptive steroids
PAR	pregnane activated receptor
PB	phenobarbital
PBREM	phenobarbitone-responsive enhancer module
PCN	pregnenolone 16 α -carbonitrile
PCR	polymerase chain reaction
PRL	prolactin receptor
PPAR	peroxisome proliferators activated receptor
PPRE	PPAR-responsive element
PR	progesterone receptor
PXR	pregnane x receptor
PXRE	PXR-responsive element
QPCR	quantitative real-time PCR
RACE	rapid amplification of cDNA ends
RAR	retinoic acid receptor
Rif	rifampicin
RT	reverse transcription, reverse transcriptase
RXR	retinoid x receptor
SHP	small heterodimerization partner
SMRT	silencing mediator of retinoid and thyroid receptors
SR12813	([3, 5- bis(1, 1- dimethylethyl)- 4- hydroxyphenyl]ethenylidene)bis-, tetraethyl ester

STAT	signal transducer and activator of transcription
SV40	simian vacuolating virus 40
SXR	steroid x receptor
TCDD	2,3,7,8-tetrachlorodibenzo- <i>p</i> -dioxin
TCPOBOP	1,4-bis[2-(3,5-dichloropyridyloxy)]benzene
Tg	transgenic
TR	thyroid hormone receptor
UDP-GA	uridinediphospho-glucuronic acid
UGT(s)	udp-glucuronosyltransferase(s)
VDR	vitamin D receptor
VP16	herpes simplex virus (HSV)-encoded transcriptional activator protein
WY-14643 / WY	4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid
XRE	xenobiotic response element

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The identification of the PPAR α -responsive element on the *UGT1A1* promoter, figures 2-5 and 2-6, are reprinted from *Drug Metabolism and Disposition* 2007, Senekeo-Effenberger K, Chen S, **Brace-Sinnokrak E**, Yueh MF, Bonzo JA, Argikar U, Kaeding J, Trottier J, Remmel RP, RitterJK, Barbier O, Tukey RH. Expression of the human *UGT1* locus in transgenic mice by Wy-14643 and implications on drug metabolism through PPAR α activation. **35** (3):419-27. I identified the PPRE through promoter-transfection studies, constructed the concatenated PPRE-reporter, and performed the luciferase experiments.

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3. “Tissue-Specific Control of the *UGT1* Locus by the Pregnane X Receptor” at Experimental Biology 2008 (ASPET division), San Diego, CA, April 2008.
4. “Induction of *UGT1* Genes by the Fibrate WY-14643 in Tg-*UGT1* Mice and Identification of Peroxisome Proliferators Activated Receptor alpha (PPAR α) Responsive Elements on the *UGT1A1* and *UGT1A4* Promoters” at Experimental Biology 2007 (ASPET division), Washington, DC, May 2007.
5. “Hormonal Regulation of the Human *UDP-Glucuronosyltransferase-1* (*UGT1*) Locus During Pregnancy and Lactation in *UGT1* Transgenic Mice” at the International Union of Basic and Clinical Pharmacology Meeting, Beijing, China, July 2006.
6. “Hormonal Regulation of Human *UDP-Glucuronosyltransferase-1* (*UGT1*) Expression During Pregnancy and Lactation in Reproductive Organs of Transgenic *UGT1* mice” at the Southern California Society of Toxicology Meeting, Irvine, CA, September 2005.
7. “The Generation of *UGT1* Transgenic Mice” at the 11th International Glucuronidation Workshop, Dundee, Scotland, October 2004.

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2. Senekeo-Effenberger K, Chen S, **Brace-Sinnokrak E**, Bonzo JA, Yueh MF, Argikar U, Kaeding J, Trottier J, Remmel RP, Ritter JK, Barbier O, Tukey RH. Expression of the human UGT1 locus in transgenic mice by 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid (WY-14643) and implications on drug metabolism through peroxisome proliferator-activated receptor alpha activation. *Drug Metab Dispos*. 2007 Mar;35(3):419-27
3. **Brace-Sinnokrak E**, Evans RM, Tukey RH. The Role of the Pregnane X Receptor as a Tissue-Specific Master Regulator of Human UDP-Glucuronosyltransferase-1 Expression in Transgenic Mice. *Manuscript*.
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ABSTRACT OF THE DISSERTATION

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and the Role of the Pregnane X Receptor as a Tissue-Specific Master Regulator

by

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Doctor of Philosophy in the Biomedical Sciences

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Professor Robert H. Tukey, Chair

The UDP-glucuronosyltransferases (UGTs) represent the most significant family of Phase II drug metabolism enzymes and assist in the detoxification and removal of endogenous and exogenous compounds from the body. Using UDP-glucuronic acid as a cosubstrate, the UGTs catalyze the transfer of a polar glucuronic acid moiety to a substrate, thereby increasing its size and hydrophilicity, facilitating excretion. The UGTs are subject to regulation by several nuclear receptors (NRs), including the aryl hydrocarbon receptor (AhR), pregnane x receptor (PXR), constitutive androstane receptor (CAR), and peroxisome-proliferators activated receptor (PPAR). Using a Tg-*UGT1* mouse line that expresses all nine functional human UGT1A proteins, it was demonstrated that many of the *UGT1* genes are regulated by these nuclear receptors in vivo. In ex vivo primary Tg-*UGT1* hepatocyte cultures, PPAR α was shown to regulate *UGT1A1*, and promoter analysis experiments were undertaken to identify a PPAR α -responsive element on the *UGT1A1* promoter.

A DR1 element at -3272 was characterized as the PPAR α -responsive sequence. *UGT1A4* was also shown to be upregulated by the nuclear receptors in vivo. Experiments to characterize nuclear receptor response elements in the *UGT1A4* regulatory region resulted in the identification of an AhR-responsive sequence at -3086 and a PPAR α -responsive sequence at -366. A putative PXR-responsive element was identified at -212, and although deletion past or mutation of this element resulted in loss of PXR inducibility, basal transcriptional levels increased dramatically, suggesting that in the absence of an agonist, PXR may act through this element to suppress transcription.

Regulation of the UGTs during hormonal events such as development and pregnancy has been well demonstrated in humans and rats, and pregnant Tg-*UGT1* mice exhibit dramatic upregulation of UGT1A protein during pregnancy. Although the hormonal changes that occur during the human menstrual or the mouse estrus cycles are much less severe than in pregnancy, *UGT1* regulation was shown to occur during estrus. PXR was hypothesized to be the mediator of the hormonal regulation, and experiments were undertaken to examine the contribution of PXR to *UGT1* induction. A Tg-*UGT1*-PXRnull mouse line was generated and was demonstrated to have no functional PXR. It was expected that induction during pregnancy and estrus would be lost in the absence of PXR, however, induction was maintained. Basal levels of *UGT1* transcription were elevated in the Tg-*UGT1*-PXRnull mice, and it was hypothesized that this could be due to the impact of PXR on cross-talk with other nuclear receptors. Induction studies were performed in Tg-*UGT1* and Tg-*UGT1*-PXRnull mice using CAR and AhR agonists. Induction of CAR and AhR control

genes was unaffected by the loss of PXR, but basal expression and inducibility of the *UGT1* genes were dramatically different in the PXRnull background.

CHAPTER 1
INTRODUCTION

The Importance of Drug Metabolism

The process of drug metabolism encompasses detoxification and recycling of critical endogenous compounds as well as exogenous toxicants, dietary constituents, and pharmaceutical drugs. Drug metabolism genes evolved over millennia in response to changing environmental and dietary conditions: early prokaryotes existed in a highly oxidized environment and possessed relatively simple reductive functions that allowed for survival, while the human body today must protect against assault by a wide array of xenobiotic chemicals from food and the environment as well as endogenous compounds that must be cleared to maintain homeostatic health, and thus have evolved a highly complex and multiple-ordered system of oxidative, reductive, hydrolytic, and conjugative reactions that allow for detoxification of this expansive set of structurally divergent compounds (Danielson, 2002; Loomis WF, 1988; Nebert and Dieter, 2000). Most of these compounds are lipophilic, allowing for diffusion through the lipid-rich cell membranes and access to their sites of action inside the cells. Drug metabolizing enzymes facilitate structural modifications that render these compounds more hydrophilic; these hydrophilic metabolites are more easily removed from cells, are directed toward the water compartments in the body, and are ultimately excreted through the urine and bile.

In higher eukaryotes, drug metabolism and transport can be classified into three phases. Phase I metabolism is characterized by small polar modifications such as oxidation, reduction, hydroxylation, peroxidation, and hydrolysis, which add or reveal hydrophilic groups on the compound. These changes can be sufficient to direct the metabolite to the urine for excretion or act as a substrate for Phase II reactions,

but they may also act as reactive species on the molecule which can interact with critical cellular macromolecules and lead to toxicity and carcinogenicity (Danielson, 2002; Miller and Miller, 1981). Thus, while Phase I metabolism is a largely detoxification event, important active or toxic metabolites may emerge. In Phase II metabolism, compounds with reactive groups, including Phase I metabolites, are further detoxified through addition of large polar moieties such as glucuronic acid, glutathione, glucose, methyl and sulfate groups, and amino acids, resulting in compounds that are highly water soluble and can be excreted in the urine or bile. Phases I and II work in concert toward the ultimate detoxification of endogenous and exogenous compounds and elimination from the body and are therefore essential to human health.

Discovery and Characterization of UDP-Glucuronosyltransferases

Of the Phase II metabolism processes, glucuronidation is the most significant and represents the detoxification pathway for the majority of pharmaceutical drugs as well as many endogenous compounds such as bilirubin (Ritter et al., 1991), steroids (Belanger et al., 1998; Hum DW et al., 1999; Levesque et al., 2001), and bile acids (Kirkpatrick et al., 1984; Trottier et al., 2006). The process of glucuronidation is catalyzed by the UDP-Glucuronosyltransferases, which utilize uridine diphosphoglucuronic acid (UDP-GA) as a cosubstrate and catalyze the transfer of the glucuronic acid moiety to form a glucuronide metabolite. Glucuronides were first characterized in the urine of animals: by Schmid in 1855, who isolated and identified euxanthic acid, a glucuronide, in the urine of cattle that had fed on mango leaves (DUTTON,

1980), by Schmiedeberg and Meyer, who in 1879 identified a camphor metabolite in dog urine as a glucuronide (Schmiedeberg O and Meyer H, 1879), and by Quick in 1924 who identified a rapidly excreted menthol glucuronide in rabbits (Quick A.J., 1924). Although it was elucidated that glucose is a direct precursor of the glucuronic acid moiety on glucuronide metabolites (Mosbach and KING, 1950), the mechanism for formation of the glucuronic acid intermediary was unclear until Dutton and Storey in 1951 extracted a thermostable cofactor required for the formation of a phenol glucuronide (DUTTON and STOREY, 1951). Subsequent analysis identified this cofactor as uridine diphosphoglucuronic acid and revealed its role as a cosubstrate in the formation of glucuronides (DUTTON and STOREY, 1953; DUTTON and STOREY, 1954). The enzymes responsible for catalyzing this reaction, the UDP-Glucuronosyltransferases (UGTs), were initially localized to liver homogenates (DUTTON and STOREY, 1954), then to the microsomal membrane fraction of cells (ISSELBACHER, 1956; DUTTON, 1980), and eventually to the endoplasmic reticulum (Roy *et al.*, 1986). Although it was accepted that lipid was an essential component for transferase function (Berry *et al.*, 1978; Tukey *et al.*, 1979), the question of topological orientation in the membrane remained controversial. Microsomal preparations demonstrate a so-called “latency” in that full activity cannot be achieved without physical or chemical disruption of the microsomal vesicles (DUTTON, 1975; DUTTON and Burchell, 1977). One hypothesis to explain this phenomenon asserted that the catalytic core of the transferase exists on the luminal side of the endoplasmic reticulum, with the membrane acting as a physical barrier between it and the substrates, cofactors, and nucleotide sugar cosubstrate; disruption

of the membrane allows interaction among the reaction components (Hallinan and De Brito, 1981). Another theory placed the catalytic core of the transferase enzyme on the cytoplasmic side of the ER but in a constrained conformation that prevented activity. According to this theory, perturbation of the membrane causes an unnatural relaxed conformation and artificially high activity. Studies by Vanstapel and Blanckaert (Vanstapel and Blanckaert, 1987; Vanstapel and Blanckaert, 1988a; Vanstapel and Blanckaert, 1988b), Berry and Hallinan (Berry and Hallinan, 1976), and Shepherd et al (Shepherd *et al.*, 1989) presented strong evidence for the luminal-core model. Sequence analysis and hydropathy plotting years later eventually led to the general acceptance of this model of UGT membrane topology.

During this time, the promiscuous nature of the UGT enzyme was becoming apparent as glucuronides of a growing number of structurally divergent substrates were being identified. A debate over the possible multiplicity of the UGTs emerged, with competing theories of either a single UGT with broad substrate specificity (AXELROD et al., 1957; Mulder, 1971) or a set of UGT enzymes with differing but overlapping substrate specificities (DUTTON, 1966a; Wishart, 1978a; Wishart, 1978b). The recognition that differences in activity exist during development or in different tissues (DUTTON, 1966a; DUTTON, 1966b; DUTTON, 1978) was viewed by some as evidence for multiplicity, while others asserted that the divergent activities were due to variations in membrane microenvironment and subsequent effects on the UGT active site (Mulder, 1971). A breakthrough came in 1975 when Del Villar et al chromatographically resolved activity toward different substrates, resulting in two species of enzymes labeled morphine-UGT and p-nitrophenol-UGT

(Del Villar *et al.*, 1975). This method was verified by Bock *et al.* in the separation of activity toward morphine and 1-naphthol (Bock *et al.*, 1977). The technique was further refined, first by use of consecutive ion-exchange columns (Burchell, 1977), and then by Gorski and Kasper's UDP-hexanolamine sepharose affinity chromatography method (Gorski and Kasper, 1977), which allowed for isolation of enzymes with significantly higher specific activity than was obtained by previous methods. Gorski and Kasper also noted that full activity was absolutely dependent on lipid, and further studies demonstrated that activity of delipidated enzyme could be restored upon addition of phospholipid mixtures (Gorski and Kasper, 1978; Tukey and Tephly, 1980; Tukey *et al.*, 1979). Although these refined techniques allowed for the purification of substrate-specific UGTs such as oestrone-UGT (Tukey *et al.*, 1978) and testosterone-UGT (Weatherill and Burchell, 1980), it became clear that these methods were often not sufficient to purify a UGT to homogeneity. In 1983, Falany and Tephly first employed isoelectric focusing in combination with UDP-hexanolamine sepharose chromatography to separate the isoenzymes responsible for steroid glucuronidation at the 3-hydroxy versus 17-hydroxy positions (Falany *et al.*, 1983; Falany *et al.*, 1986; Falany and Tephly, 1983), and this method was later used by Roy Chowdhury *et al.* (Roy *et al.*, 1986) to isolate six distinct isoenzymes. Isoelectric focusing (chromatofocusing) in combination with either anion-exchange or affinity chromatography soon became the method of choice for isoenzyme purification.

Although it had become apparent that multiple UGT isoenzymes with overlapping substrate specificities existed, the number and degree of homology was still unclear. Peptide mapping and amino acid comparison of 4-nitrophenol-UGT, 3 α -

hydroxysteroid-UGT, and 17 β -hydroxysteroid-UGT showed a high degree of homology between the hydroxysteroid-conjugating enzymes, but 4-nitrophenol-UGT was significantly different from either, suggesting that two or more UGT subfamilies may exist (Falany *et al.*, 1986). But it was not until UGT mRNA and cDNA clones were identified in the late 1980s that a full appreciation for the sequence similarities and homologies among the UGT genes could be ascertained. Antibodies raised against purified UGT protein preparations allowed for immunoprecipitation of nascent UGT protein associated with its polysomes and mRNA. In 1984, three UGT clones were identified by Mackenzie, Gonzalez, and Owens from a cDNA library constructed from Phenobarbital-induced rat liver mRNA isolated by this method. Two clones showed high nucleotide sequence identity and mapped by Southern Blot to overlapping regions of the genome; the third clone was less similar to the other two (Mackenzie *et al.*, 1984). A study by Jackson and Burchell a year later identified several UGT clones from a rat liver cDNA library and segregated the clones into two subfamilies based on sequence homology (Jackson *et al.*, 1985). Together these studies provided direct nucleotide sequence evidence for UGT multiplicity and the existence of at least two distinct families of isoenzymes. Cloning, sequencing, and identification of rodent UGT genes followed: UDPGTr-2F, known today as Ugt2b1 (Mackenzie, 1986), UDPGTr-4 / androsterone-UGT (Ugt2b2) (Jackson and Burchell, 1986; Mackenzie, 1986a), 17 β -hydroxysteroid-UGTs UDPGTr-3 (Ugt2b3) (Harding *et al.*, 1987; Mackenzie, 1986a) and UDPGTr-5 (Ugt2b6) (Mackenzie, 1990), and the 4-nitrophenol-UGT (Ugt1a6) (Harding *et al.*, 1988). Human UGTs were cloned as

well: H1ugP1 (UGT1A6) (Harding *et al.*, 1988), H1ug25 (UGT2B4), and H1ug6 (UGT2B7) (Fournel-Gigleux *et al.*, 1989).

During this time, as numerous rodent, human, and rabbit UGTs were being cloned, a centralized system of nomenclature was devised that classified the enzymes based on sequence homology within and among species. This is largely the system used today and it reflects the evolutionary divergence within the UGT superfamily, resulting in the definition of two subfamilies, *UGT1* and *UGT2* (Burchell *et al.*, 1991; Mackenzie *et al.*, 2005; Mackenzie *et al.*, 1997; Tukey and Strassburg, 2000). The *UGT2* genes are clustered on chromosome 4q13-q21 and encode three UGT2A and seven UGT2B proteins (Monaghan *et al.*, 1994). The UGT2Bs, including UGT2B4, 2B7, 2B10, 2B11, 2B15, 2B17, and 2B28, glucuronidate primarily endogenous compounds, including steroids and hormones (Belanger *et al.*, 1998; Guillemette *et al.*, 2004; Hum DW *et al.*, 1999; Jin *et al.*, 1997; Lepine J *et al.*, 2004; Levesque *et al.*, 2001) as well as bile acids (Monaghan *et al.*, 1997; Pillot *et al.*, 1993; Turgeon *et al.*, 2003b), but can also conjugate xenobiotics and therapeutic drugs (Coffman *et al.*, 1998; Green *et al.*, 1994; Turgeon *et al.*, 2003a). The UGT2A family has only more recently been cloned and characterized. UGT2A3, interestingly, appears to be specifically active toward bile acids and is expressed in the liver and gastrointestinal tract (Court MH *et al.*, 2008; Tukey and Strassburg, 2001), while UGT2A1 and 2A2 are most highly expressed in the olfactory epithelium and have activity toward androgens, xenobiotics, and odorant compounds (Jedlitschky *et al.*, 1999; Strausberg *et al.*, 2002). UGT2A1 and 2A2 are also structurally distinct among the UGT2 family: while UGT2A3 and the UGT2Bs exist as individual genes

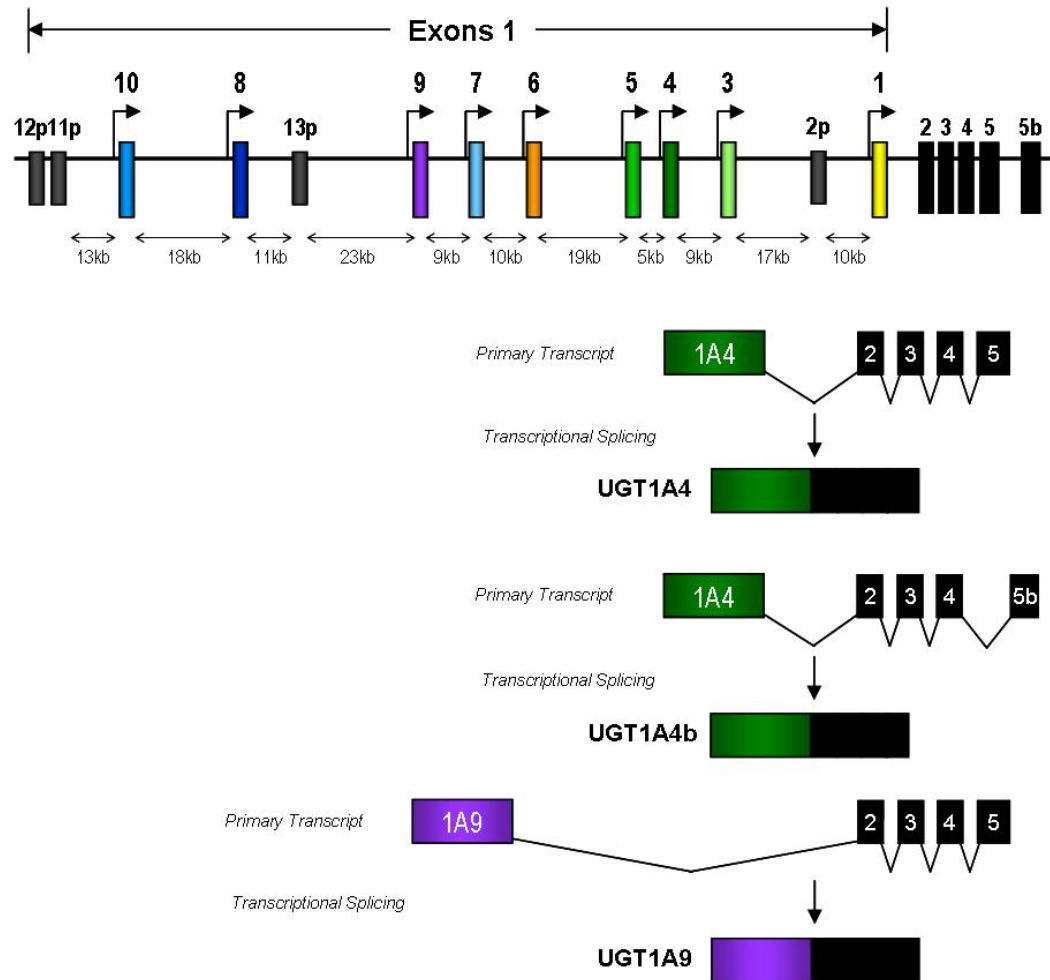


Figure 1-1. Organization of the Human *UGT1* Gene Locus. The locus, spanning 220 kilobases on chromosome 2q37, contains 13 cassette exons 1 that encode the 5' half of individual *UGT1* transcripts and a single complement of exons 2-5/5b. There are nine functional exons 1 and four pseudogenes (p). Each exon 1 is under control of individual regulatory regions and promoter elements, allowing for independent RNA transcription. Each exon 1 contains a 3' splice site that is spliced directly to the 5' splice site of exon 2; intervening exons 1 are treated as introns and are spliced out during RNA processing. As each primary transcript yields one specific product, this process is referred to as "exon sharing" and not "alternative splicing." Adapted from Gong *et al*, 2001.

clustered together on chromosome 4, UGT2A1 and 2A2 are products of exon sharing, wherein individual 2A1 or 2A2 exon 1 sequences under control of individual promoter regions are spliced to shared exons 2 through 6, creating gene products that have identical 3' sequences (Court MH et al., 2008). However, this gene structure, though relatively uncommon in humans (Li and Wu, 2007; Zhang et al., 2004), is not unique within the UGT superfamily. In 1992, Ritter et al recognized that the 3' ends of several UGT cDNA clones shared 100% nucleotide identity and described a gene locus (Figure 1-1), involving several exon 1 elements spliced individually to a shared set of exons 2-5, that would produce the gene products observed. This locus included six 5' exons 1 with individual regulatory elements (Ritter et al., 1992a), and was later extended 5'-ward to include seven additional exon 1 regions (Gong et al., 2001). Of the thirteen exon 1 regions identified, four (*UGT1A2*, *1A11*, *1A12*, and *1A13*) are pseudogenes, while the other nine (*UGT1A1*, *1A3-10*) result in functional proteins with unique N-terminal and identical C-terminal sequences (Mackenzie et al., 1997). Recently, a distinct exon 5 (5b) downstream of the original exon 5 (5a) was identified, and resultant splice variants of UGT1A1 and 1A3-10 were identified (*UGT1A1_i2*, etc) (Levesque *et al.*, 2007). These splice variants are distributed throughout many tissues and, though enzymatically inactive, may act in concert with the 5a variants to modulate activity (Girard et al., 2007), though any in vivo significance of this is yet unknown.

Although the liver had long been considered the predominant organ of drug metabolism, early microsomal preparations from other human tissues also demonstrated a capacity for glucuronidation. Organs of the gastrointestinal tract, the

site of absorption for most dietary and xenobiotic compounds, produced glucuronides of bile acids (Matern et al., 1984;Parquet et al., 1988;Parquet et al., 1985) and bilirubin (McDonnell et al., 1996;Pacifici et al., 1988;Peters and Jansen, 1988;Peters et al., 1991;Peters et al., 1989). Glucuronidation was also identified in kidney microsomes (Matern et al., 1984;Parquet et al., 1988), but it was unclear to what extent extrahepatic glucuronidation contributed to overall clearance of endogenous or exogenous compounds. Development of methods to purify UGT protein to homogeneity enabled generation of antibodies; however, the exceptionally high degree of amino acid homology among certain members of the UGT1A family, for example UGT1A3, 1A4, and 1A5 (>93% homology) or 1A7, 1A8, and 1A10 (>89% homology) (Tukey and Strassburg, 2000) has prevented the generation of isoenzyme-specific antibodies for several UGT1As. A lack of specific antibodies coupled with overlapping substrate specificities meant that it was not possible to determine the exact complement of UGT expression within each tissue through immunohistochemical or protein analysis.

The development of reverse-transcriptase polymerase chain reaction and the high fidelity synthesis and purification of specific short oligonucleotide primers made it possible for the first time to distinguish between single nucleotide differences in highly homologous sequences and selectively amplify UGT1A mRNA transcripts (Strassburg *et al.*, 1997). This made it possible to detect all expressed UGT1A transcripts in multiple tissues, including those in which expression levels are low or are localized to a small percentage of total cells in the sample. The human liver expresses UGT1A1, 1A3, 1A4, 1A5, 1A6, and 1A9 (Finel et al., 2005;Strassburg et

al., 1997a;Strassburg et al., 1997b) but not 1A7, 1A8, or 1A10, which is interesting given the extensive capability for these isoforms to glucuronidate a wide range of steroids, therapeutic drugs, phenolic compounds, and dietary flavanoids (King *et al.*, 2000). UGT1A8 and 1A10 are, however, widely expressed extrahepatically, especially in the intestines, and UGT1A7 and 1A10 are highly expressed in the stomach and esophagus. The small and large intestines, indeed, represent the most complete complement of UGT1A expression of all studied human tissues (Strassburg et al., 1997a;Strassburg et al., 1998;Strassburg et al., 1997b;Strassburg et al., 1999;Tukey and Strassburg, 2001), and this expression likely plays an essential role in nonhepatic glucuronidation of consumed substances. The study of other organs revealed significant UGT expression outside of the gastrointestinal tract: UGT1A9 was identified in human kidney (McGurk *et al.*, 1998), where it may function to detoxify compounds upon reabsorption in the proximal tubules. UGT1A6 was identified in neurons of the rat brain (Martinasevic *et al.*, 1998) and human cerebellum (King et al., 1999), and was shown to efficiently glucuronidate the neurotransmitter serotonin (King et al., 1999), providing evidence for an important role for glucuronidation in this organ. UGT1A7 and 1A10 are highly expressed in the aerodigestive tract, where they likely protect against airborne carcinogens such as those found in cigarette smoke and auto exhaust (Zheng et al., 2002a;Zheng et al., 2001a). UGT1A expression has also been detected in steroid target tissues (Chouinard *et al.*, 2006) as well as human heart (Ohno and Nakajin, 2009).

Glucuronidation and Human Health and Disease

The wide distribution of UGTs throughout the body underlies the importance of glucuronidation to human health. Though classically perceived as a mechanism for clearance of therapeutic drugs, glucuronidation by the UGTs is also a critical pathway for protection against environmental and dietary toxic compounds, and plays an important role in homeostatic regulation of endogenous molecules. Substrates for the UGTs vary widely in size and structure, and the large number of UGT isoenzymes has likely evolved to address conjugation of such a diverse field of compounds. Although substrate specificities tend to overlap (Tukey and Strassburg, 2000), there are many examples of important molecules or classes of molecules that are specifically glucuronidated by one transferase (Barbier and Belanger, 2008; Barbier et al., 2000; Hum et al., 1999; Ritter et al., 1992b; Ritter et al., 1992c; Trottier et al., 2006). Therefore, altered expression or activity of drug metabolism enzymes can have serious clinical implications, both in maintenance of normal human health and also in pharmaceutical therapy.

UGT1A1 is the sole enzyme responsible for detoxification and clearance of bilirubin, the product of heme catalysis (Bosma *et al.*, 1994). Unconjugated bilirubin circulates in the plasma, bound to serum albumin; without conjugation, saturation of albumin occurs and eventually causes bilirubin deposition in tissues. At very high concentrations, unconjugated bilirubin can cross the blood-brain barrier, causing kernicterus, a fatal condition characterized by necrosis of neurons and glial cells in the brain (Wiener and Brody, 1946). A less severe form of unconjugated hyperbilirubinemia is characterized by neonatal and adult jaundice: the Gunn rat was described as a model for this disease in 1938 (Gunn C.H., 1938; Malloy H.T and

Lowenstein L, 1940). It was later determined that these rats lacked the enzyme required for production of bilirubin glucuronide (LATHE and WALKER, 1957;LATHE and WALKER, 1958), and in 1989 the Gunn rat UGT1A1 cDNA sequence revealed a single base pair deletion in the shared exon region that results in a C-terminal truncation of the protein, yielding an inactive enzyme (Iyanagi et al., 1989). In humans, clinical unconjugated hyperbilirubinemia is classified by the severity of the disease: Crigler-Najjar syndrome type I (CN-I), Crigler-Najjar type II (CN-II), and Gilbert's syndrome. CN-I and CN-II both result from mutations in the promoter and coding regions of the *UGT1A1* gene, though with different consequences (Seppen et al., 1994). CN-I, characterized by often lethal levels of unconjugated serum bilirubin, is due to a near complete absence of bilirubin glucuronidation and is caused by mutation of a strictly conserved di-glycine motif in the UGT1A1 protein that renders it inactive (Ciotti et al., 1998;Ciotti et al., 1995). Clinically, phototherapy can be used to facilitate bilirubin catabolism, but the only curative therapy is liver transplantation (Al-Shurafa *et al.*, 2001). CN-II is a more treatable form of hyperbilirubinemia. Unlike CN-I, the mutations in the promoter and coding regions of the *UGT1A1* gene result in lower expression and partial activity of the protein, and CN-II patients typically have serum bilirubin levels in an elevated but not acutely toxic range (Kadacol et al., 2000;Kadacol et al., 2001;Seppen et al., 1994). These patients are often successfully treated with chemicals that induce UGT1A1 expression, such as Phenobarbital (Arias *et al.*, 1969), alone or in combination with phototherapy. Gilbert's syndrome is the mildest form of hyperbilirubinemia and is considered relatively harmless; it is also the most common,

with 6-12% of the population affected (Black and Billing, 1969; Monaghan et al., 1996). Gilbert's is typically associated with a fully functional UGT1A1 protein under control of a promoter with a TA insertion in its TATAA element which results in reduced efficiency of transcriptional initiation and a 30-50% reduction in UGT1A1 expression (Bosma et al., 1995). Homozygosity for the TA repeat variant allele does not appear to be the sole factor contributing to Gilbert's, however. Several other mutations in the coding regions of the *UGT1A1* gene have been identified and statistically associated with mild unconjugated hyperbilirubinemia (Aono et al., 1995; Kadakol et al., 2001; Koiwai et al., 1995; Sava and Kraemer, 2005). Genetically, it appears that several mutations the *UGT1A1* gene and promoter contribute to the phenotype. Clinically, slightly elevated bilirubin levels and mild jaundice may be the only symptoms of Gilbert's, though significant complications can arise in patients under pharmacotherapy with compounds that utilize UGT1A1 as a major pathway for clearance.

One such drug is the chemotherapeutic agent irinotecan. Irinotecan is biotransformed into its active metabolite, SN-38, which is cleared primarily through glucuronidation by UGT1A1 (Iyer et al., 1998). Irinotecan, like most cancer chemotherapeutic agents, has a low therapeutic index and close monitoring of dosage is required to minimize toxicity. Impaired clearance results in increased exposure and toxic side effects, including severe diarrhea resultant from the effects on intestinal epithelia (Gupta et al., 1994). Individuals homozygous for Gilbert's alleles are at an increased risk for this toxicity (Iyer et al., 2002; Wasserman et al., 1997). Because Gilbert's occurs at a relatively high percentage in the average population and most

patients are otherwise asymptomatic, *UGT1A1* genetic markers can serve as effective predictors of toxicity in irinotecan therapy.

The variability of UGT1A1 expression and activity also has implications in the metabolism and toxicity of acetaminophen. Acetaminophen is cleared primarily through glucuronidation by UGT1A1 (Bock et al., 1993;Hjelle and Klaassen, 1984), UGT1A6, and 1A9 (Court MH et al., 2001). When glucuronidation is impaired, either by genetic modulation of UGT expression or activity or through competition for the glucuronidation pathway with co-administered or endogenous compounds, pools of unglucuronidated acetaminophen are bioactivated by Phase I enzymes to a toxic metabolite. This metabolite is normally cleared through conjugation with glutathione (de Moraes and Wells, 1988). High concentrations of the metabolite, however, quickly deplete glutathione, resulting in accumulation of a metabolite capable of interacting with macromolecules in cells, particularly those in the liver and kidney, causing damage and possible carcinogenesis (Jollow et al., 1973;Mitchell et al., 1973a;Mitchell et al., 1973b;Potter et al., 1973). Polymorphic forms of UGT1A1, 1A6, and 1A9 have been associated with increased risk for acetaminophen toxicity (Court MH et al., 2001;Gagne et al., 2002;Tankanitlert et al., 2007;Villeneuve et al., 2003;Zheng et al., 2001b), supporting the role for glucuronidation as a protective mechanism in the human body.

The role of interindividual variation among the UGTs in carcinogenesis and cancer is just beginning to be understood. Drug metabolism is the body's defense against environmental carcinogens, and altered drug metabolism profiles may present serious deficiencies that can lead to increased susceptibility to these toxic compounds.

UGT1A7, present in many extrahepatic tissues including those of the aerodigestive tract, is highly active toward the so-called “tobacco smoke carcinogens,” including benzo(*a*)pyrene, and constitutes a first line of defense in the organs of exposure (Fang et al., 2002;Zheng et al., 2002b). Like most of the UGTs, UGT1A7 is highly polymorphic and significant differences in activity of different polymorphic forms of the enzyme toward benzo(*a*)pyrene have been observed (Guillemette *et al.*, 2000). Zheng et al in 2001 demonstrated that polymorphic alleles associated with low activity strongly correlated with increased risk for oral and laryngeal cancers, both in smokers and non-smokers (Zheng et al., 2001b). The presence of UGT1A7 in nonhepatic organs of the gastrointestinal tract also led researchers to examine the effect of polymorphisms on common dietary carcinogens toward which 1A7 is active, and low-activity 1A7 alleles were found to correlate with risk of hepatic and colorectal cancer, particularly when consumption of carcinogenic heterocyclic amines was examined (Kuchenbauer et al., 2004;Ockenga et al., 2003;Strassburg et al., 2002;Vogel et al., 2001;Vogel et al., 2002;Wells et al., 2004). It appears that mutations in UGT1A7 are not alone enough to cause a carcinogenic outcome, but in the context of environmental and dietary exposure to toxic substrates, low-activity alleles present a risk factor for cancer.

Exposure of the human body to environmental, dietary, and pharmaceutical compounds does not constitute the only pathway, however, for cancer. The growth and development of many cancers are stimulated by hormones in men and women. The UGTs play an important role in homeostatic regulation of hormone levels through glucuronidation of androgens (Chouinard et al., 2007;Chouinard et al.,

2008;Hum et al., 1999;Lepine J et al., 2004) and estrogens (Guillemette et al., 2004;Lepine J et al., 2004), and alterations in UGT activity could affect systemic and local hormone concentrations. UGT1A1 is an important component of the estradiol clearance pathway and is expressed in human mammary tissue. Low-activity UGT1A1 alleles correlated with occurrence of invasive breast cancer and elevated serum estradiol levels in African-American women (Guillemette et al., 2000a), though these alleles did not correlate with endometrial cancer (McGrath *et al.*, 2009). As increased circulating estrogen levels are linked with increased incidence of breast cancer, increased androgen concentrations correlate with prostate cancer. UGT2B15 is the primary pathway for androgen clearance in the prostate and controls local cellular exposure to the hormone (Chouinard *et al.*, 2008). Interestingly, patients with a nonvariant allele appear to have a higher risk for prostate cancer than those with a high-activity polymorphism, indicating that polymorphic mutations are not necessarily deleterious to human health (MacLeod *et al.*, 2000).

An essential component to human health, glucuronidation exists as a protective mechanism, facilitating excretion of pharmaceutical agents, environmental contaminants, or endogenous compounds and preventing accumulation of molecules that can lead to toxicity and cancer.

Xenobiotic Regulation of the UGTs

In addition to tissue-specific and humoral factors that govern differential expression throughout the body, expression of the UGTs is highly regulated by xenobiotics. This regulation, coupled with the large number of allelic polymorphisms

known to exist in the UGTs, determines an individual's glucuronidation profile and susceptibility to toxic insult and cancer. It also explains the large range of activities toward drugs and xenobiotics observed in the population that are not explained by genetics alone. Early studies on UGT regulation focused on liver-enriched transcription factors such as the family of hepatocyte nuclear factors (HNFs). HNF1 α was first shown to be essential for rat UGT2B1 expression (Hansen et al., 1997), and investigation of human UGTs revealed HNF1 responsive elements on most *UGT1A* promoters. *UGT1A1* is activated by both HNF1 α and HNF1 β (Bernard et al., 1999), and HNF1 α has been shown to regulate *UGT1A3* and *1A4* (Gardner-Stephen and Mackenzie, 2007b), *UGT1A8*, *1A9*, and *1A10* (Gregory et al., 2004), *UGT2B7* (Radomska-Pandya et al., 2001), and *UGT2B17* (Gregory et al., 2000) through direct binding to conserved HNF1 palindromic response elements on the promoters. Sequences of *UGT2B4*, *UGT2B10*, *UGT2B11*, and *UGT2B15* promoters have revealed similar conserved HNF1 consensus sites as well (Mackenzie et al., 2003; Turgeon et al., 2000). HNF4 α , a related liver-enriched transcription factor, is a positive regulator of *UGT1A1* and *1A6* (Aueviriyavit et al., 2007; Kamiyama et al., 2007) and has been shown to interact with a consensus site on the *UGT1A9* promoter (Barbier et al., 2005), though activation by HNF4 α appears to be dependent on HNF1 (Gardner-Stephen and Mackenzie, 2007a), indicating that the liver-enriched transcription factors may participate in a complex network of cross-regulation, further modifying an individual's expression of drug metabolizing enzymes.

Animals evolved this regulatory process to ensure proper basal expression of enzymes required to detoxify and clear endogenous compounds from the body. Each

individual, however, is exposed to a different set of environmental and dietary compounds and must be able to respond in a transient and effective way to these insults. Mammals have evolved a set of ligand-dependent transcription factors to address the need for inducible expression of drug metabolism genes, and these nuclear receptors, including the steroid receptors, adopted orphan nuclear receptors, and the Aryl Hydrocarbon receptor, have been implicated in the regulation of the *UGT1* and/or *UGT2* genes.

The Aryl Hydrocarbon Receptor

The Aryl Hydrocarbon Receptor (AhR) was first characterized as the mediator of induction of aryl hydroxylase activity by the polycyclic aromatic hydrocarbons (PAHs) (Nebert and Gelboin, 1968a; Nebert and Gelboin, 1968b). Genetic studies on varying aryl hydroxylase activity mapped the genes encoding this mediator to a locus, termed the *Ah* locus (Gielen et al., 1972; Poland et al., 1974) or the *Ahh* gene (Thomas et al., 1972), and this gene product was later described as a receptor that modulates the expression of the aryl hydroxylase enzyme (Guenthner and Nebert, 1977; Nebert et al., 1975), known today as the Phase I metabolism Cytochrome P450 CYP1A1 (Owens and Nebert, 1975; Tukey et al., 1981). The AhR is activated by compounds with a range of chemical structures, the best characterized of which are the polycyclic aromatic hydrocarbons (PAHs), namely benzo(*a*)pyrene (B[*a*]P), and the halogenated aromatic hydrocarbons (HAHs), among which is the most potent AhR agonist, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Poland and Glover, 1973; Poland and Glover, 1974).

The unliganded AhR exists in the cytosol as part of a multimeric complex with the heat shock protein HSP90 and other proteins (Denis et al., 1988;Perdew, 1988;Perdew, 1992). Hydrophobic ligands enter the cell through diffusion and bind to the complex, causing a conformational change that unmask the DNA-binding domain and a nuclear localization signal. The ligand-bound AhR/HSP90 complex is shuttled into the nucleus, where HSP90 is exchanged for the Ah Receptor Nuclear Translocator (Arnt) (Petrulis and Perdew, 2002), and the AhR/Arnt heterodimer recognizes and binds to specific DNA sequences known as Dioxin Responsive Elements (DRE) or Xenobiotic Responsive Elements (XRE), resulting in transactivation of target genes such as CYP1A1 (Hoffman et al., 1991;Probst et al., 1993;Reyes et al., 1992). The consensus sequence for the DRE/XRE, 5'-T/GNGCGTG-3', is bound by both AhR and Arnt proteins (Luska et al., 1993;Probst et al., 1993): AhR binds the 5' T/GNGC half site, while Arnt recognizes the more conserved 3' GTG sequence (Swanson et al., 1995). Both proteins have a transactivation domain in their C-terminal sequence that recruits coactivators and ultimately the transcriptional machinery (Kikuchi et al., 2003).

Activation by AhR has been described for several Phase I and Phase II enzymes, and clearly constitutes a pathway for coordinate clearance of harmful toxicants. Many of the PAHs are bioactivated by Phase I metabolism through CYP1A1 to the ultimate carcinogen: an example of this is the conversion of benzo[*a*]pyrene to B[*a*]P 7,8-dihydrodiol-9,10-epoxide-2, the most carcinogenic of the B[*a*]P metabolites (Conney, 1982). These metabolites are ultimately cleared through Phase II conjugation. In addition to CYP1A1 and CYP1A2 (Hines et al.,

1988;Quattrochi et al., 1994), the AhR is capable of upregulating several Phase II genes, including *UGT1*. In transgenic mice expressing the *UGT1* locus, TCDD treatment resulted in induction of every *UGT1* isoform in the liver or intestines (Chen S et al., 2005). Thus far, XREs have been identified on the *UGT1A1* (Yueh et al., 2003), *UGT1A3* (Lankisch et al., 2008), *UGT1A4* (Erichsen et al., 2008), and *UGT1A6* (Bock et al., 1999;Munzel et al., 1998) promoters.

The Adopted Orphan Nuclear Receptors

The nuclear receptors (NRs) are ligand-dependent transcription factors that have important roles in biological processes, including cell proliferation, differentiation, and homeostasis (Mangelsdorf and Evans, 1995). Among the NR family are steroid receptors, such as the Estrogen, Progesterone, and Glucocorticoid receptors, which are activated by small steroid ligands. The adopted orphan nuclear receptors are also a subclass of the nuclear receptor family, but differ from the steroid receptors in several ways. Despite a high degree of structural similarity between the steroid and adopted orphan subfamilies (Jacobs et al., 2003;Kumar and Thompson, 1999;Thompson and Evans, 1989), they are distinguishable by a highly conserved portion of the DNA binding domain called the P box: receptors that possess a larger P box are categorized as type II nuclear receptors, which include the adopted orphans, whereas small P box-containing receptors are considered type I and include the steroid receptors. The dimerization and DNA binding processes also differ significantly: steroid receptors form homodimers almost exclusively and bind to direct repeat halfsites (AGGTCA or ACAACA) separated by three nucleotides

(Glass, 1994;Kumar and Thompson, 1999), whereas the type II adopted orphan NRs are able to form homodimers (Forman et al., 1989;Noble et al., 2006) but most often form heterodimers with the Retinoid X receptor (Kliewer et al., 1992a). Additionally, ligands for the adopted orphan receptors are more varied in size and structure, and are not exclusively endocrine (Enmark and Gustafsson, 1996).

The characterization of the Retinoid X receptor (RXR) in the early-to-mid 1990s revealed its role as an essential component for specific binding and activation by the subset of nuclear receptors later termed the adopted orphan NRs (Kliewer et al., 1992a;Mangelsdorf and Evans, 1995;Mangelsdorf et al., 1990), which include the Thyroid Hormone Receptor (TR) (Forman et al., 1989), Retinoic Acid Receptor (RAR) (Kliewer et al., 1992a;Yu et al., 1991), Vitamin D Receptor (VDR) (Kliewer et al., 1992a;Yu et al., 1991), Farnesoid X Receptor (FXR) (Forman et al., 1995;Seol et al., 1995), Liver X Receptor (Willy et al., 1995), Peroxisome Proliferators Activated Receptor (PPAR) (Kliewer et al., 1992b), Constitutive Androstane Receptor (CAR) (Baes *et al.*, 1994), and the Pregnane X Receptor (PXR) (Blumberg et al., 1998;Kliewer SA et al., 1998). The heterodimers recognize AG(G/T)TCA halfsites with a spacing of one to eight nucleotides and can recognize direct repeat (DR), everted repeat (ER), and/or inverted repeat (IR) halfsites (Carlberg et al., 1993;Frank et al., 2003;Mangelsdorf and Evans, 1995;Sueyoshi and Negishi, 2001;Tini et al., 1993;Umesono et al., 1988;Umesono et al., 1991), depending on the receptor (Figure 1-2B).

Structurally, the adopted orphan NRs are highly related; prior to the identification of their ligands or function, they were classed together because of the

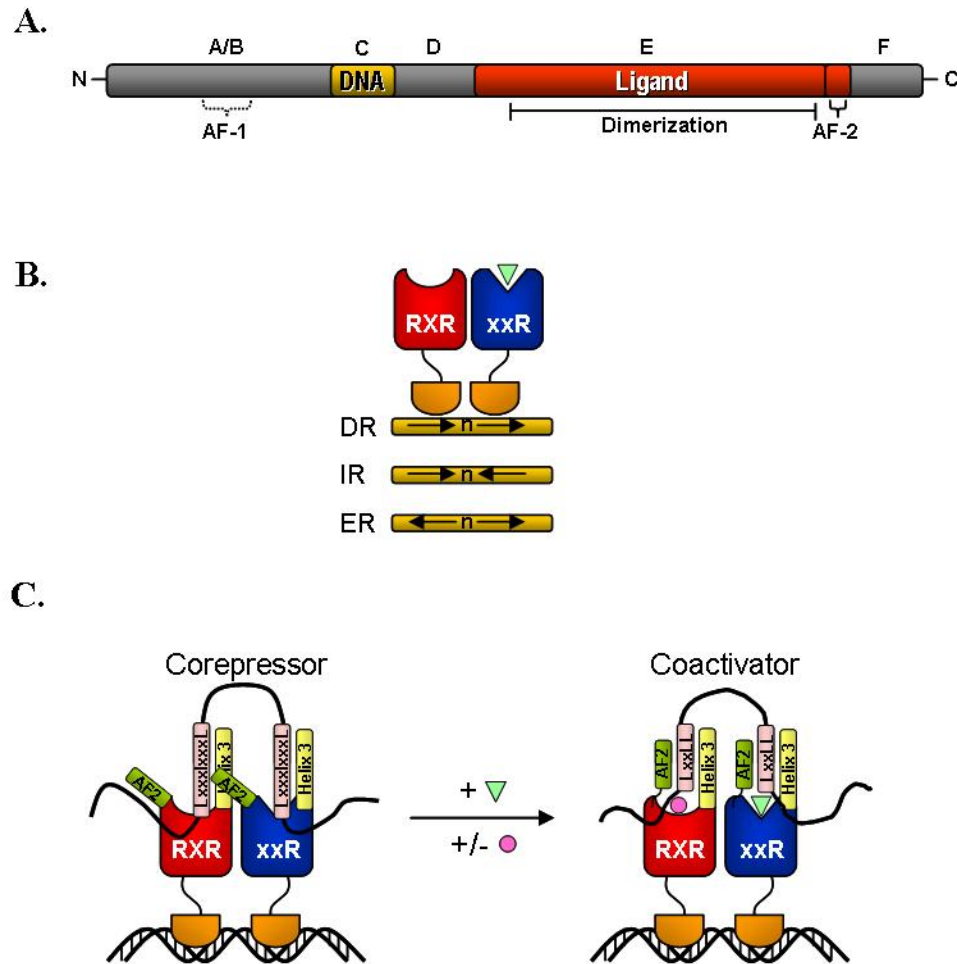


Figure 1-2. Adopted Orphan Nuclear Receptor Structure and Function. **A)** The adopted orphan nuclear receptors are comprised of five functional regions (A-F), including a DNA-binding domain (C), a ligand-binding and dimerization domain (E), and two activation domains, AF1 and AF-2. A second dimerization domain (not shown) is contained within domain C and is required for heterodimerization on DNA response elements. **B)** Nuclear receptor DNA response elements are composed of repeats of the consensus AG(G/T)TCA core sequence. These can be direct repeats (DR), inverted repeats (IR), or everted repeats (ER). Specificity for the RXR-xxR heterodimer is directed by the number of nucleotides (n) between the repeats. **C)** Model for coregulator exchange of DNA-associated NR heterodimers. The corepressor LxxxIxxxL/I motif or the coactivator LxxLL motif associates with Helix 3 of the NR. Binding of an agonist ligand is associated with movement of the AF2 domain to stabilize these contacts. Adapted from Mangelsdorf and Evans, 1995 and Glass and Rosenfeld, 2000.

high degree of homology. The receptors all have four main domains: an N-terminal and a C-terminal activation domain (AF-1 and AF-2, respectively), a DNA-binding domain (DBD), and a ligand binding / dimerization domain (LBD) (Figure 1-2A). Additional residues within the DBD are also required for proper heterodimerization and binding to response elements (Evans, 1988; Green and Chambon, 1988). The DBD is the most conserved region among these already highly similar proteins, spanning 66 residues in the N-terminal half of the proteins and containing eight cysteines that form a pair of zinc finger motifs. These zinc fingers ensure proper folding of the protein and enable an alpha helix, termed the “recognition” helix, to contact the nucleotides of the half site within the major groove of the DNA helix (Luisi *et al.*, 1991). RXR and its heterodimerization partner both interact with their recognition sequence through this alpha helix motif, binding in adjacent major grooves, with RXR occupying the 5' half site (Rastinejad *et al.*, 1995). The LBD, a 225 amino acid domain in the N-terminal half of the protein, is not only important for ligand recognition but also contains essential domains for dimerization. The domain is largely comprised of 3 sets of stacked antiparallel alpha helices that form a pocket. Within the pocket is a ligand dependent transcriptional activation domain (Weigel, 1996). The stacked alpha helices of the LBD also act as the dimerization face, creating a large surface for interaction with RXR (Evans, 1988; Green and Chambon, 1988; Kumar and Thompson, 1999; Mangelsdorf and Evans, 1995). The DBD and LBD work in concert, enabling RXR to heterodimerize with a number of other receptors while recognizing nearly identical DNA sequences: the spacing of the DR, ER, or IR half sites determines the receptor specificity. An increase of a single

nucleotide in the spacer causes a significant rotation of the heterodimerization partners such that unique residues in the LBD alpha helices make contact in each protein pair (Mangelsdorf and Evans, 1995). This enables this class of structurally similar nuclear receptors that bind to a common heterodimerization partner and interact with a common AG(G/T)TCA half site to nonetheless translate a diverse set of physiological and exogenous signals and cause induction of a number of drug metabolism genes. Immediately C-terminal of the LBD is the AF-2 activation domain, which serves as a molecular switch by interacting with coactivators or corepressors (Figure 1-2C) (Glass and Rosenfeld, 2000;Polly et al., 2000;Rachez and Freedman, 2000), enabling the recruitment of histone acetylases or deacetylases and subsequent transcriptional activation or repression.

The influence of this class of NRs on control of drug metabolism genes continues to be explored. Of particular interest are nuclear receptors that are responsive to xenobiotics in addition to their endogenous ligands. PPAR, activated by endogenous fatty acids and central in the control of lipid homeostasis, is also a target for many pharmaceutical drugs prescribed to combat hyperlipidemia, whereas CAR and PXR both act as xenosensors for a large set of structurally diverse compounds, including many pharmaceutical drugs, and act on many Phase I, II, and III drug metabolism genes.

The Peroxisome Proliferators Activated Receptor

Peroxisome proliferators are a group of compounds that cause the growth and proliferation of peroxisomes (Lock et al., 1989;Reddy and Mannaerts, 1994), cellular

organelles that contribute to the oxidation of fatty acids. In rodents, peroxisome proliferation is detrimental, leading to liver hyperplasia, hepatomegaly, and carcinogenesis (Hess et al., 1965; Issemann and Green, 1990; Green et al., 1992), and it has been postulated that increased fatty acid oxidation results in increased H_2O_2 and leads to DNA damage and subsequent tumor formation (Rao and Reddy, 1989). Enzymes that catalyze the β -oxidation and ω -hydroxylation of fatty acids are upregulated by peroxisome proliferators, including the Phase I CYP4A family (Lazarow and De, 1976; Sharma et al., 1988; Muerhoff et al., 1992a; Gibson et al., 1982), and increased fatty acid oxidation can also be elicited by a high-fat diet and other dietary factors. The receptor mediating this induction was discovered to be highly related to the thyroid/retinoid/adopted orphan receptor family, and was termed the peroxisome proliferator activated receptor α (PPAR α) (Muerhoff et al., 1992b; Kliewer et al., 1992).

PPAR α was first cloned from mouse liver and was demonstrated to be responsive to multiple peroxisome proliferator compounds (Issemann and Green, 1990) and fatty acids (Gottlicher et al., 1992; Gearing et al., 1994). Human (Sher et al., 1993) and rat (Kliewer et al., 1992; Gottlicher et al., 1992) PPAR α cDNAs were subsequently cloned. Reporter studies with promoters of peroxisome proliferator- or fatty acid-inducible genes demonstrated that PPAR α , coupled with RXR, mediates induction of these genes, including *acyl CoA-oxidase*, rat *HMG CoA-reductase*, rabbit *CYP4A6*, and its rat homologue *Cyp4a1* (Tugwood et al., 1992; Issemann et al., 1992; Marcus et al., 1993; Muerhoff et al., 1992b; Aldridge et al., 1995). Furthermore, it was demonstrated that targeted disruption of the PPAR α gene in mice prevents the

carcinogenic and pathological changes resulting from exposure to peroxisome proliferator compounds, confirming the role of PPAR α in this pathway (Lee et al., 1995). Additional members of the PPAR family (PPAR γ and PPAR δ / PPAR β) were cloned in several species and it was demonstrated that these isoforms were only weakly activated by peroxisome proliferators but could be activated by other non-PPAR α ligands (Kliewer et al., 1994; Tontonoz et al., 1994; Dreyer et al., 1992; Chen et al., 1993; Zhu et al., 1993). These three isoforms are expressed in a tissue-specific manner: PPAR α is expressed predominantly in the liver and intestines and to a lesser extent in the heart and muscle whereas PPAR γ is expressed primarily in adipose tissue and PPAR δ is expressed in very low levels in the liver but abundantly throughout the rest of the body (Kliewer et al., 1994; Evans et al., 2004). This variable pattern of expression of the three isoforms coupled with their divergent ligands provides insight into the different roles each receptor may perform in activation of target genes. Indeed, the expression of PPAR γ in adipocytes underlies its central role in glucose homeostasis and lipid storage and metabolism (Evans et al., 2004), while PPAR α expression in the liver controls fatty acid oxidation, providing energy during fasting and preventing fatty acid accumulation (Kersten et al., 1999; Reddy and Hashimoto, 2001). The importance of these receptors to lipid metabolism, homeostasis, and storage as well as to insulin sensitization has made the PPARs logical targets for pharmaceutical development. Thiazolidinediones, drugs to combat insulin desensitization in diabetic patients, are ligands for PPAR γ , while PPAR α is targeted by the important class of anti-hyperlipidemic drugs, the fibrates.

The pathological effects of peroxisome proliferators were well-studied in rodent and models in the 1990s. However, it was clear that these findings did not hold true in humans, who do not develop liver disease in response to peroxisome proliferator compounds (Lock et al., 1989), suggesting a species-divergent PPAR response. Comparison of cloned PPAR α revealed high homology between the human and mouse receptors, with 100% homology of the DBD and 94% homology of the LBD (Sher et al., 1993), indicating that any species-divergent activity of the receptor is likely not due to differences in PPAR response element recognition, and is instead due to species-specific affinity for ligands or differences in the complement of or sequences of target genes and promoters. Studies using cloned hPPAR α either transfected into mouse cell lines or expressed in humanized mice showed that, upon treatment with peroxisome proliferator compounds, hPPAR α was able to induce mPPAR α target genes (Macdonald et al., 1999; Cheung et al., 2004; Yang et al., 2008). Furthermore, no differences were noted in the ability for two prototypical PPAR α ligands, WY-14643 (WY) and fenofibrate, to induce target genes in the hPPAR α humanized or wild type animals, indicating that neither response element recognition nor ligand specificity are central to the species-divergent pathological response to peroxisome proliferators. Interestingly, however, PPAR α humanization was sufficient to drastically reduce hepatocyte proliferation and hepatomegaly in the mouse (Morimura et al., 2006). It is proposed that this is due to a species-specific ability for mPPAR α to induce cell cycle control genes and oncogenes that may instigate proliferation, such as c-Myc, CD24, Anxa2, CD39, and Ly6D; these genes are not induced in hPPAR α -humanized mice (Yang et al., 2008; Cheung et al., 2004).

Thus, the PPAR α signaling pathway appears to be conserved between mice and humans, and the species-divergent reaction to treatment with peroxisome proliferators may have evolved in humans as a protective mechanism against these compounds, many of which are man-generated environmental contaminants such as phthalate ester plasticizers and solvents as well as herbicides, pharmaceutical drugs, and synthetic hormones.

Both human and mouse PPAR α -RXR heterodimers interact with DNA response elements that are imperfect direct repeats of AGGTCA separated by one nucleotide (DR1). The first PPAR α responsive element (PPRE) was characterized in 1992 as direct repeats TGACCT and TGTCCCT with a single nucleotide spacer; the element was identified on the promoter of the prototypical PPAR-responsive gene Acetyl CoA Oxidase (Tugwood et al., 1992). Shortly thereafter, Muerhoff et al identified a PPRE on the *CYP4A6* promoter that was similar in sequence to that on Acetyl CoA Oxidase (Muerhoff et al., 1992b), and this discovery was followed by the characterization of two more PPRE sequences on the *4A6* promoter by the same group (Palmer et al., 1994). It was determined that the middle element, termed the Z element, was the predominant response element on the promoter, with the distal and proximal elements having less specific affinity for the PPAR α -RXR heterodimer. A DR1 site allows the binding of not only PPAR α -RXR but also RXR homodimers and RAR-RXR heterodimers. The nuclear receptors ARP1 and COUP-TF can also bind to the PPRE and act as repressors (Nakshatri and Chambon, 1994; Miyata et al., 1993), illustrating that spacing alone is not sufficient to confer specific binding by PPAR α -RXR. Palmer et al demonstrated in 1995 that sequences immediately 5' of

the DR1 were required for transactivation, and alignment studies by the same group of known PPRE sequences revealed a conserved 5' extension of the PPRE consensus site that is believed to allow for preferential binding to PPAR α -RXR heterodimers over RAR-RXR or RXR homodimers (Johnson et al., 1996). These results were further refined to distinguish the exact nucleotide requirements for PPAR α , PPAR γ , or RXR binding (Juge-Aubry et al., 1997;Castelein et al., 1997).

The Constitutive Androstane Receptor

The characterization of a novel human orphan nuclear receptor in 1994 was notable because the receptor, upon dimerization with RXR, transactivated a subset of retinoic acid receptor response elements without the addition of an exogenous ligand (Baes et al., 1994). A related mouse receptor that possessed the same ability to transactivate response elements in the absence of ligand was characterized in 1997 and named the constitutive activator of retinoid response (CAR) (Choi et al., 1997), later shortened to constitutive active receptor and today called the constitutive androstane receptor owing to the first endogenous ligands identified (androstanol and androstanol) (Forman et al., 1998).

Phenobarbital, an anesthetic used frequently in the first half of the twentieth century, was first described as an inducer of drug metabolism in 1960 (Conney et al., 1960), and subsequent studies identified the Phase I cytochrome P450s and Phase II UDP-glucuronosyltransferases as the phenobarbital (PB) responsive enzymes (Leighton et al., 1984;Waxman et al., 1985;Mackenzie et al., 1984). CYP2B enzymes, in particular, were highly induced by PB and structurally related

compounds (Okey, 1990;Lubet et al., 1992;Honkakoski et al., 1996), and in the mid-1990s mouse *Cyp2b10* was characterized as the predominant PB-responsive cytochrome P450 (Honkakoski et al., 1996;Honkakoski and Negishi, 1997). The creation of two strains of transgenic mice possessing the rat *Cyp2b2* gene (homologue to the mouse *Cyp2b10* and human *CYP2B6*) and varying lengths of upstream regulatory sequence revealed that the Phenobarbital-responsive element was not in the proximal promoter sequence but rather farther upstream in the enhancer module (Ramsden et al., 1993). This led to the identification of a PB-responsive element (PBREM) on the mouse *cyp2b10*, rat *cyp2b2*, and human *CYP2B6* regulatory sequence, localized to a 51-bp site approximately 2.3 kilobases 5' of the gene (Trottier et al., 1995;Honkakoski and Negishi, 1998;Park et al., 1996;Sueyoshi et al., 1999). The presence of a *trans*-acting regulatory element was an unexpected finding, as most xenobiotic-mediated regulatory sequences had, until this point, been associated with the few hundred base pairs directly 5' of the gene.

Characterization of the nuclear receptor CAR soon revealed its role as the mediator of PB induction. Of the two putative nuclear receptor response element half sites identified on the mouse *cyp2b10* PBREM, NR1 and NR2 (Honkakoski and Negishi, 1997;Honkakoski et al., 1998a), CAR-RXR was found to associate with NR1 (Honkakoski et al., 1998b), a DR4 element. Identification of CAR-RXR DR4 response elements on other PB-inducible drug metabolism genes rapidly followed, including, notably, UGT1A1 (Sugatani et al., 2001;Xie et al., 2003) as well as several other Phase I, II, and III metabolism genes.

The mechanism by which CAR exerted its effects, however, remained unclear. In vitro, co-translation of CAR and RXR enabled transactivation of response elements without ligand (Baes et al., 1994), but in vivo studies required an agonist such as PB to elicit gene induction. Paradoxically, the endogenous ligands for which the receptor was named actually suppressed target gene transcription: 3 α -hydroxy, 5 α -reduced androstanes were capable of dissociating co-activators from CAR (Forman et al., 1998; Sueyoshi et al., 1999) and repressing transcription of reporter genes. This repression was lifted upon treatment with PB and transcription was induced (Sueyoshi et al., 1999), suggesting that CAR, in cooperation with endogenous ligands, can function as a suppressor, but can also respond to xenobiotic ligands and become a transcriptional activator. This suppressor / activator duality was novel among the nuclear receptors, and implied an important hepatoprotective role for CAR, which was further demonstrated by development of a CAR-null mouse line. These mice did not exhibit PB induction of the prototypical CAR target genes such as *Cyp2b*. Interestingly, many genes were downregulated by PB treatment in wild type but not CAR-null mice. These genes encoded enzymes involved in basic liver function, such as fatty acid oxidation, energy metabolism, cell surface communication, transcriptional processes, embryogenesis, and tissue repair. This dual regulation by a xenobiotic may provide a way for the cells to cope with toxic insult and prevent toxicity. Unexpectedly, a few genes were *only* inducible by PB in the CAR-null mice, including the *Cyp4a* genes, which encode lipid peroxidases. This presents a possible third role for CAR as a non-PB-responsive suppressor, serving to

protect the cells against oxidative damage in response to xenobiotic insult (Ueda et al., 2002).

The comparison of induction profiles in wild type and CAR-null mice also revealed a large subset of genes that were regulated by PB in a CAR-independent manner (Ueda et al., 2002), making PB an effective but nonspecific CAR activator. Additionally, PB acts through an unknown indirect mechanism, causing CAR translocation into the nucleus without binding the receptor directly (Kawamoto et al., 1999). Two planar xenobiotics, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) and 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO), were found to be potent CAR activators that binds competitively to the receptor's ligand binding domain and are specific mCAR (TCPOBOP) or hCAR (CITCO) agonists (Tzameli et al., 2000; Maglich et al., 2003); these compounds are the most potent CAR-specific agonists found to date and are considered prototypical mCAR or hCAR ligands.

Structurally, CAR is similar to the other adopted orphan NRs, containing DBD, LBD, and AF2 domains. The N-terminal AF1 domain is smaller in mCAR and hCAR than in other related nuclear receptors, though the effect of this is still unclear. Unlike the other NRs, CAR can be activated by xenobiotics (PB) in an indirect manner not involving residues of the ligand binding domain. Choi et al showed in 1997 that removal of residues within the AF2 domain of hCAR abrogated its ability to transactivate (Choi et al., 1997) but later studies proved that the AF2 domain was not required for PB-induced nuclear translocation (Zelko et al., 2001), demonstrating that CAR translocation and transactivation are regulated by different mechanisms.

Translocation activity was instead mapped to a C-terminal LXXLXXL sequence. The finding that TCPOBOP, which binds to mCAR but not to hCAR, caused translocation of both receptors, indicates a ligand-independent translocation mechanism (Zelko et al., 2001), which may involve phosphorylation of the C-terminal motif (Kawamoto et al., 1999). Another unique feature of CAR is its ability to transactivate target genes in vitro without a ligand. In general, unliganded nuclear receptors were believed to exist in the cytosol; association with a repressive ligand resulted in a corepressor complex on target gene response elements. The presence of activating ligand would displace the inhibitory ligand and structural changes would allow for an exchange of corepressor and coactivator proteins [reviewed in (Glass and Rosenfeld, 2000)]. Although it had been established that the unliganded thyroid hormone and retinoic acid receptors can exist in a corepressor complex on target genes (Hu and Lazar, 1999; Hu et al., 2001), unliganded activation of transcription had not previously been observed. TR and RAR associate with corepressors through a coregulator cleft. In other unliganded nuclear receptors, this cleft is masked by the AF2 domain. Ligand binding induces a conformational change that reorients the AF2 domain to allow for coactivator association; for TR and RAR, this change also displaces the corepressors (Zhang et al., 1999). Interestingly, in CAR, the coregulator cleft and AF2 domains are conserved, and it is the presence of a short loop between the LBD and AF2 and a short C-terminal helix that allows the AF2 domain to dock into a groove on the LBD, causing CAR to assume a ligand-activated conformation (Dussault et al., 2002; Andersin et al., 2003; Suino et al., 2004). Thus, repression by inhibitory ligands such as androstanol involves displacement of the AF2 domain from

the ligand binding pocket, dissociation of coactivators, and recruitment of corepressors. The persistent presence of the physiological inhibitor in vivo likely accounts for the reduced constitutive activity compared to that observed in vitro.

The identification of CAR as the mediator of many planar PB-like xenochemicals and the discovery of its ability to induce a breadth of detoxification enzymes has led to CAR being classified as a xenosensor, however it is clear that CAR is also central to the regulation of many homeostatic and hepatoprotective processes.

The Pregnane X Receptor

Originally identified based on sequence homology with other known nuclear receptors, a novel NR was cloned and characterized in 1998 by three groups who ascribed names to the receptor based on initial characterizations of its ligands. The Steroid and Xenobiotic Receptor (SXR) (Blumberg et al., 1998), Pregnane “X” Receptor (PXR) (Kliewer SA et al., 1998), and Pregnane Activated Receptor (PAR) (Bertilsson et al., 1998) were later designated NR1I2 in the standard nomenclature (1999), though it is most commonly referenced as PXR in the literature. The importance of this NR to drug metabolism was established early on with the discovery of its ability to regulate the *Cyp3a* genes. In humans, CYP3A4 is the primary phase I drug metabolizing enzyme expressed in the liver, where it catalyzes the modification of up to 50% of all pharmaceutical drugs (Guengerich, 1999). In addition to having broad substrate specificities, the CYP3A family is also notable because it is induced by a variety of structurally diverse chemicals, many of which are detoxified by the CYP3As in a feedback loop (Denison and Whitlock, Jr., 1995).

As it became clear that this regulatory pathway could underlie serious drug-drug interactions, much attention was focused on characterization of the mediator of the regulatory event.

PXR was identified in the liver, intestines, colon, and to a lesser extent in the kidney and lung in humans as well as laboratory rodents (Blumberg et al., 1998; Kliewer SA et al., 1998). In mouse, low levels of the receptor are also expressed in female reproductive organs, including ovary, placenta, and uterus (Masuyama H et al., 2001), and PXR transcript has been identified in both normal and neoplastic human mammary tissue (Dotzlaw et al., 1999). Expression in the liver and intestines is common to most of the nuclear receptors in this family, such as PPAR and CAR. Expression in the reproductive organs, however, appears to be unique to PXR and mirrors expression of endocrine hormone receptors such as the Estrogen or Progesterone Receptors. Identification of endogenous and xenobiotic ligands for PXR further distinguished this receptor from its structurally-related brothers. Unlike other NRs, PXR is activated by a broad range of ligands diverse in size and structure [reviewed in (Jones et al., 2000; Kliewer et al., 2002)]. These include not only xenobiotics such as the antimycotic drug clotrimazole, the antidiabetic drug and PPAR α ligand troglitazone, the CAR activator Phenobarbital, the antihypertensive agent spironolactone, and the cancer chemotherapeutics tamoxifen and paclitaxel, but also naturally occurring steroids, including progesterone, estradiol, and several of their metabolites (Kliewer SA et al., 1998; Blumberg et al., 1998; Xie et al., 2000a; Lehmann et al., 1998).

Interestingly, activation of the receptor, in particular by endogenous hormones, varies widely by species. Mouse PXR is readily activated by pregnanes, whereas the human receptor is activated efficiently by estradiol. But perhaps most strikingly, a very potent activator of human PXR, Rifampicin, exhibits almost no activation of the mouse receptor, while pregnenolone 16 α -carbonitrile (PCN) activates the mouse receptor exclusively (Jones et al., 2000). Comparison of PXR sequence among species revealed that the DNA-binding domains (DBD) are highly conserved: the DBD from human and mouse receptors share more than 95% identity (Jones et al., 2000). The ligand-binding domains (LBD), however, are much more divergent than in other NR. The human and mouse LBD, for example, are only 77% homologous, compared to 94% LBD identity for PPAR α , and this disparity among LBD may account for the observed divergent activation profiles.

PXR is separated from its family members by its broad class of ligands, its tissue distribution, and its species-divergent activation. Characterization of a LBD crystal structure of in 2001 revealed additional differences that set PXR apart from the other adopted orphan nuclear receptors (Watkins et al., 2001). A 60-residue insertion between helices 1 and 3 of the LBD results in the formation of an α -helix motif and two β -sheets oriented in a β -turn- β motif that are not present in other NRs. These additional elements result in an enlarged and much more flexible domain that can accommodate ligands of varied size; the additional residues in the β -strands and α -helix can also form stabilizing contacts with polar residues on ligands. Additional crystal structures with ligands of various sizes and structures including the antidepressant hyperforin and the hypolipidemic drug SR12813 (Zhang et al.,

2001;Watkins et al., 2003), the antibiotic rifampicin (Chrencik et al., 2005), the LXR agonist T0901317 (Xue et al., 2007a), and 17 β -estradiol (Xue et al., 2007b) demonstrated that ligands of varying sizes are accommodated by the LBD and can have multiple orientations and make contact with different sets of residues to further stabilize interaction with the domain. This structural flexibility is unique to the PXR LBD, and resembles neither the conventional type I NR nor the other type II adopted orphan NR. Analysis of the LBD crystal structure also resulted in identification of specific residues within the LBD that contribute to species differences in ligand activation. Mutation of four polar residues in the mouse LBD to the corresponding residues found in the human receptor resulted in a humanized response to ligand (Watkins et al., 2001), demonstrating that despite the promiscuous nature of the PXR LBD and its ability to interact with a variety of ligands, key residues exist that determine discrete profiles of activation for the different species.

While the additional motifs in the PXR LBD confer a unique flexibility to the ligand pocket, the β -turn- β motif also contains an oligomerization interface not found in other NRs. All NR LBDs fold into a three-layer α -helical sandwich which forms the standard dimerization motif and allows for interaction with RXR (type II receptors) or homodimerization (type I receptors) (Watkins et al., 2001;Jacobs et al., 2003). Recent studies, however, have shown that in the PXR LBD, tryptophan residues within the β -turn- β motif interlock and cause formation of a stable homodimer, and this homodimerization domain is essential to proper PXR function and its ability to recruit coactivators (Noble et al., 2006). It was further determined

that the PXR homodimer binds as a functional heterotetramer with RXR on target genes (Teotico et al., 2008).

The DBD of PXR is more highly conserved across species (Jones et al., 2000), and, like the other adopted orphan NR, binds to AG(G/T)TCA consensus halfsites. Analysis of PXR-CYP3A interactions revealed that PXR binds to DR3 response elements (Goodwin et al., 1999; Kliewer SA et al., 1998) on these genes, however, PXR was also shown to activate an ER6 element on the CYP3A4 proximal promoter (Bertilsson et al., 1998; Blumberg et al., 1998) as well as DR4 and DR5 elements on Cyp2b promoters (Xie et al., 2000b) and even an ER8 element on the phase III multidrug resistance associated protein 2 (*MRP2*) gene (Kast et al., 2002), indicating that PXR is able to bind a multitude of halfsite configurations in a promiscuous manner not seen with other nuclear receptors. Thus far, however, PXR control of the UGTs has been localized to only DR3 elements on the *UGT1A1* and *1A6* promoters (Xie et al., 2003).

Because of its ability to regulate *CYP3A* and *UGT1* genes, activation of PXR can precede clinically important drug-drug interactions. Polypharmaceutical interactions have become a critical issue in health care, and predicting potential adverse drug reactions due to polypharmaceutical interaction is an important aspect of drug safety. The antibiotic rifampicin, used as a high-dose and long-term treatment for tuberculosis, is a potent human PXR agonist that causes drug-drug interactions with many compounds that are substrates for CYP3A, including the anti-HIV drug ritonavir, oral contraceptives, and the anesthetic midazolam which is commonly used as a pre-surgery anesthetic that resulted in lower effectiveness of the drug

administered during rifampicin therapy (Niemi et al., 2003; Backman et al., 1996). Upregulation of enzymes responsible for bioactivation or formation of a toxic metabolite is also associated with PXR-drug toxicity: CYP clearance of acetaminophen usually constitutes a minor pathway for the drug's clearance, through which small amounts of a toxic metabolite are formed and are quickly cleared by phase II glucuronidation or sulfation (Jollow et al., 1973; Mitchell et al., 1973a; Mitchell et al., 1973b; Potter et al., 1973). Activation of PXR leads to upregulation of the CYPs and results in accumulation of the toxic metabolite at a rate much higher than the phase II detoxification can compensate, resulting in liver toxicity.

PXR is activated not only by pharmaceutical drugs or endogenous hormones, but also by a host of dietary and nutrient chemicals. Vitamin E, an essential nutrient, is actually a class of compounds that are sub-segregated into tocopherol and tocotrienol compounds. Tocotrienols are found in cereal and rice grains and palm oil and represent an important dietary constituent (Sen et al., 2000). Tocotrienols bind to and activate PXR and cause increased expression of PXR target genes, including CYP3A. In vivo studies in which mice fed tocotrienols as dietary supplements exhibited elevated Cyp3a11 (Kluth et al., 2005) further confirm the potential for high-dose Vitamin E supplementation to affect the metabolism of drugs taken concurrently. Dietary supplementation with St. John's Wort, widely used as an herbal alternative to antidepressant drugs but also used homeopathically for a range of ailments including sleep disorder, dysentery, diarrhea, and anxiety (Kumar et al., 2000; Kumar et al., 2006), has been shown to instigate a number of drug-drug

interactions. Of the dozen or so components of St. John's Wort, hyperforin is the compound thought to affect mood disorders (Kumar et al., 2006) but is also a very potent PXR ligand (Moore et al., 2000). As a large subset of pharmaceutical antidepressant drugs are cleared by UGT1A4, which is also upregulated by PXR, self-administration of St. John's Wort in combination with pharmaceutical therapy can result in accelerated metabolism of the drug and a negative clinical outcome. Compared to polypharmaceutical interactions, homeopathic or dietary supplementation is less often considered for possible adverse outcomes when prescribing drugs, though increasing data regarding these interactions has brought this issue to light.

In addition to its ability to regulate *CYP3A* and *UGT1* genes, PXR is important to human health because of its role in maintenance of normal homeostatic processes. Bile acids (BA), the products of cholesterol metabolism, are important in digestion and nutrient absorption but cause liver toxicity at high concentrations. Lithocholic acid (LCA), one such bile acid, is detoxified through a PXR-mediated feedback loop: PXR is activated by LCA and upregulates the expression of enzymes that detoxify BA, while activated PXR also downregulates expression of *CYP7A1*, the rate-limiting step in cholesterol metabolism (Staudinger et al., 2001b). Mice lacking PXR are susceptible to liver disease and cannot protect against LCA-induced toxicity, demonstrating the importance of PXR in bile homeostasis and its role in health and disease. PXR also appears to have roles in non-drug metabolism related human health. Vitamin K₂, a critical nutrient required for blood coagulation, is a PXR ligand. Activation of PXR by vitamin K₂ results in upregulation of genes

responsible for bone formation, maintenance of bone density, assembly of collagen extracellular matrix, and osteoblast differentiation (Tabb et al., 2003; Ichikawa et al., 2006; Igarashi et al., 2007). PXR activation therefore may have consequences unrelated to drug metabolism and drug clearance, and as new roles are discovered for this unique receptor, a greater understanding of possible drug interactions with homeostatic PXR-mediated processes will aid in predicting drug safety.

Nuclear Receptor Cross-Talk

Phase I, II, and III drug metabolism work in concert to protect the body against the accumulation of potentially harmful exogenous and endogenous compounds. Interindividual expression and activity of drug metabolizing enzymes is determined in part by single nucleotide polymorphisms and inherited polymorphic expression patterns but also by complex transcriptional control by nuclear receptors. In addition to the cell- and tissue-specific regulatory factors governing enzyme expression, cross-talk and cross-regulation among the nuclear receptors may play a large role in determining an individual's overall metabolism. PXR and CAR are closely related, sharing approximately 70% homology within the DBD and 50% homology in the LBD (Blumberg et al., 1998), and were originally characterized as the mediators of *CYP3A* and *CYP2B* genes, respectively. However, the promiscuous nature of the PXR DBD allows PXR to bind and transactivate DR4 elements in the *CYP2B* promoters in both in vitro cell culture assays and in vivo mouse models (Xie et al., 2000b; Xie et al., 2000a). Conversely, CAR is able to bind to and transactivate the DR3 and IR6 response elements on the *CYP3A* promoter (Xie et al.,

2000b;Maglich et al., 2002). PXR has also been implicated in cross-regulation with CAR and AhR: PXR can upregulate AhR (Xie and Evans, 2001) and CAR (Maglich et al., 2002), but unliganded or overexpressed human PXR suppresses CAR expression (Rosenfeld et al., 2003;Saini et al., 2005), suggesting that PXR controls these genes in a highly regulated manner. AhR activation upregulates CAR expression in vivo and may provide a mechanism for increased response to the class of planar AhR ligands (Patel et al., 2007). This extended branching of gene activation after xenobiotic insult may provide a biological reason for NR cross-talk. PXR and CAR are activated by an overlapping but distinct set of ligands, including the steroid 5 β -pregnane-3,20-dione, an efficacious activator of both receptors, as well as the prototypical PXR agonists rifampicin and PCN, the glucocorticoid dexamethasone, and the prototypical CAR agonist PB. Chemicals that bind LBDs of both receptors may not elicit similar reactions, however, as evidenced by the opposing effects of clotrimazole, which activates PXR but represses transactivation by CAR (Moore et al., 2000). CAR is also considerably less promiscuous in its interactions with ligands than is PXR, likely due to its less flexible ligand pocket (Kliewer et al., 2002). Through activation of multiple NRs or through one NR's ability to activate several sets of detoxification genes, an organism enhances the protective mechanism to deal with xenobiotic insult and prevent toxicity.

The Transgenic-*UGT1* Mouse

To study the regulatory pathways involved in the expression of the *UGT1* genes, a mouse line was generated that possesses the entire *UGT1* gene locus and its

regulatory regions (Chen et al., 2005). These mice express each of the *UGT1* isoforms in a tissue-specific manner that mirrors human expression. Studies with this demonstrated that the PXR, AhR, and PPAR α pathways were evolutionarily conserved (Chen et al., 2005; Senekeo-Effenberger et al., 2007): PXR and AhR activation resulted in upregulation of each of the nine *UGT1* isoforms in either the liver or large intestine, and activation of PPAR α resulted in upregulation of liver UGT1A1, 1A3, 1A4, and 1A6 (Figure 1-3A, B). These important data demonstrate the usefulness of the Tg-*UGT1* mouse line in studying the nuclear receptor signaling pathways.

In vivo gene studies also provide the ability to examine the effect of circulating factors as well as endocrine or paracrine signaling on gene expression in a way that cannot be replicated effectively in tissue culture. It is well known that glucuronidation activity increases dramatically after birth and during development [reviewed in (DUTTON, 1978)], and there is evidence for increased maternal drug clearance by glucuronidation during pregnancy (Bologa et al., 1991; Tran TA et al., 2002; Anderson, 2005) and in women taking oral contraceptives (Sabers A et al., 2003; Miners JO et al., 1984; Liu HF et al., 1991; Miners JO et al., 1983). Interestingly, glucuronidation rates decrease during pregnancy in rats, concordant with lower UGT1A levels in the maternal livers compared to non-pregnant animals (Luquita MG et al., 2001). This species divergent regulation makes rodents an unfavorable preclinical model system for drug metabolism studies during pregnancy. The Tg-*UGT1* mice, however, exhibit striking upregulation of the human enzymes during pregnancy and lactation (Figure 1-3C) (Chen et al., 2005), and therefore

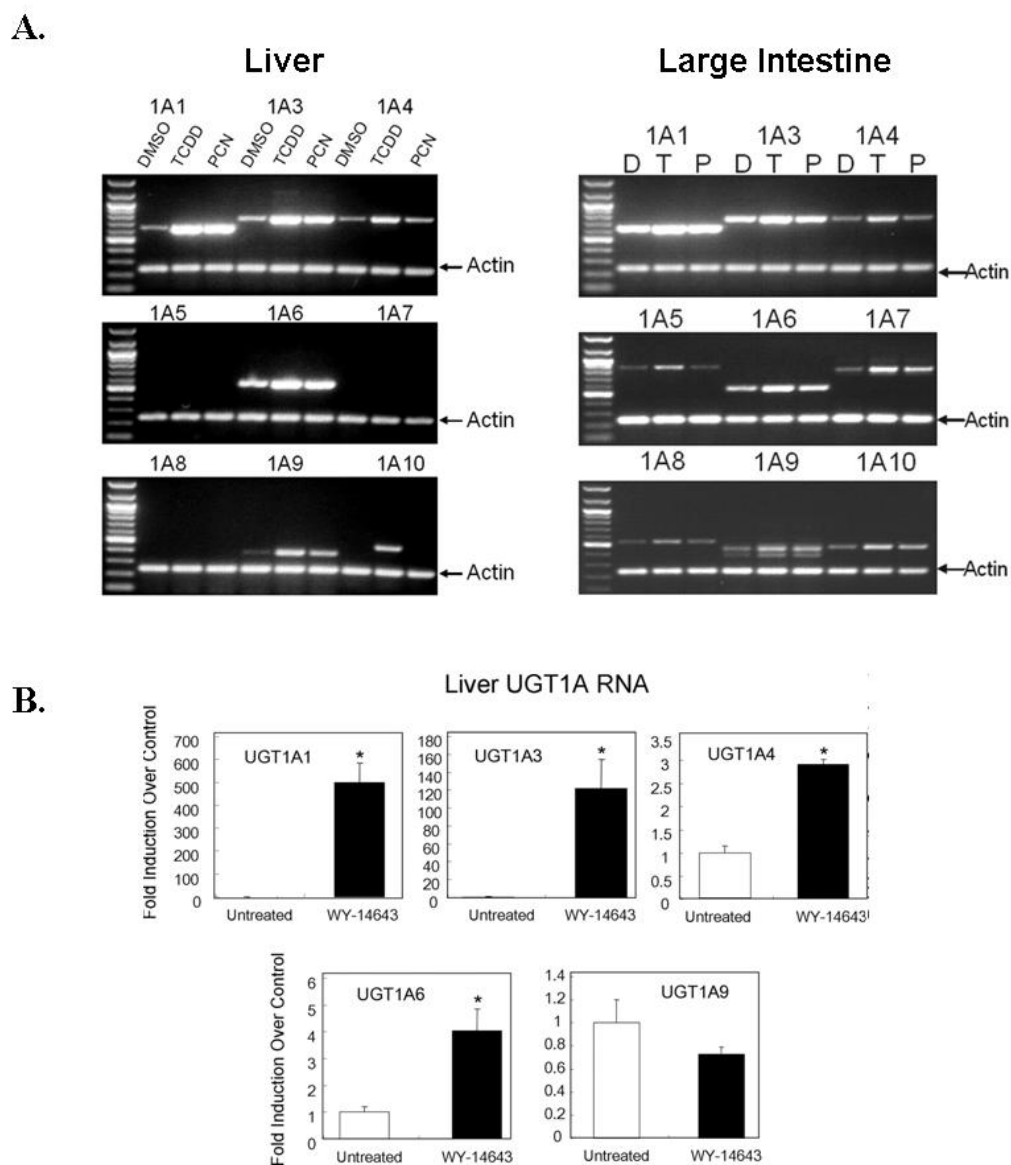


Figure 1-3. The Tg-UGT1 Mouse Model Demonstrates Conserved Regulation of the Human *UGT1* Genes. **A)** Induction of the human *UGT1* genes through conserved nuclear receptor signaling pathways is maintained in the Tg-*UGT1* mice. All nine isoforms were induced by the AhR ligand TCDD (T) and the mPXR ligand PCN (P) compared to vehicle alone (DMSO, D) in the liver or large intestine as demonstrated by RT-PCR. **B)** Tg-*UGT1* liver UGT1A1, 1A3, 1A4, and 1A6 transcript are upregulated by the mPPAR α ligand WY-14643 as demonstrated by quantitative RT-PCR.

C.

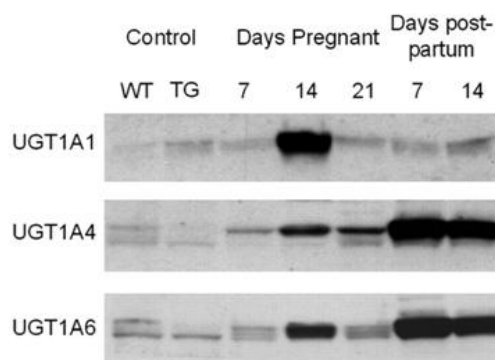


Figure 1-3, continued. C) Female Tg-*UGT1* mice display significant upregulation of liver UGT1A1, 1A4, and 1A6 protein during pregnancy and lactation. Reprinted from Chen *et al*, 2005 (A,C) and Senekeo-Effenberger *et al*, 2007 (B).

provide a unique model system with which to study UGT regulation during these hormonal events.

Objectives of the Dissertation

At the start of my dissertation work, the molecular mechanisms for UGT1 regulation by the nuclear receptors were just beginning to be understood. It was known that *UGT1A1* was regulated by PXR, CAR, and AhR through a distal nucleus of characterized response elements known collectively as the PBREM (Xie et al., 2003; Sugatani et al., 2001; Yueh et al., 2003) and a PXR-responsive element had been identified on the *UGT1A6* promoter as well (Xie et al., 2003). Chemical treatment of laboratory rodents with PXR, CAR, and AhR agonists demonstrated induction of many rodent Ugt isozymes, and cultured human cell lines such as HepG2 or primary human hepatocytes also demonstrated that the human *UGT1* genes were regulatable by these xenochemicals.

During this time, a transgenic mouse containing a chromosomally-integrated *UGT1* locus was in development in the laboratory. I participated in the initial characterization of these mice (Chen et al., 2005), determining tissue-specific expression patterns of the individual UGT1 transcripts. Induction experiments revealed that each UGT1 transcript was inducible by PXR and AhR, providing new evidence for the regulation of several *UGT1* genes by these nuclear receptors. This mouse line was further used to characterize UGT1A control by the nuclear receptor PPAR α (Senekeo-Effenberger et al., 2007). I contributed to this work through analysis of PPAR α induction in primary Tg-*UGT1* mouse hepatocytes and through identification of a PPAR α -response element in the *UGT1A1* PBREM.

Inducibility studies with PXR, AhR, and PPAR α all demonstrated that UGT1A4 is highly induced in vivo. As glucuronidation by UGT1A4 is the primary pathway for the clearance of several classes of commonly used pharmaceutical drugs, including antihistamines, tricyclic antidepressants, and antipsychotics (Chaudhuri et al., 1976; Luo et al., 1991b; Luo et al., 1991a; Luo et al., 1995; Lehman et al., 1983), the potential for adverse drug outcomes is increased when UGT1A4 levels change. I undertook experiments to identify NR-responsive elements on the *UGT1A4* promoter in order to gain a better understanding of the signal transduction pathways that govern UGT1A4 expression.

The Tg-*UGT1* mice also provided a unique model system with which to study the effect of global hormonal changes on UGT1 expression. Pregnancy studies in which liver UGT1A levels were examined in mice at early, middle, or late term gestation revealed dynamic induction during the second half of pregnancy (Chen et

al., 2005). This discovery led me to two questions. Firstly, if the hormonal changes during pregnancy are sufficient to upregulate the UGT1As so dramatically, could the smaller but still significant hormone fluxuations observed during mouse estrus cause changes in UGT1A expression, and secondly, what is the most likely mediator of this induction phenomenon? The *UGT1* genes are not known to be direct targets for control by the type I nuclear hormone receptors, including the estrogen and progesterone receptors. Both classes of steroids have, however, been shown to effectively activate PXR at concentrations lower than those observed during mid-to-late-gestation. PXR is also highly upregulated in the mouse liver during pregnancy in a temporal fashion that correlates with the increased UGT1A expression (Masuyama H et al., 2001), and we have demonstrated that PXR can regulate each *UGT1* isoform. I therefore hypothesized that PXR was the primary mediator of UGT1 induction during pregnancy, and would also mediate any observed regulation of *UGT1* genes during estrus.

To test this hypothesis, a line of PXRnull mice was obtained (Xie et al., 2000a) and was bred into our Tg-*UGT1* line. After several generations, A Tg-*UGT1*-PXRnull line was obtained, in which the human *UGT1* genes are expressed in a PXRnull background. These mice and Tg-*UGT1* mice were monitored during their estrus cycles and were selectively sacrificed at different estrus stages to obtain liver RNA samples. The Tg-*UGT1* mice demonstrated estrus-stage-dependent regulation of the *UGT1* genes; this regulation was not, however, abrogated in Tg-*UGT1*-PXRnull mice. Additional studies in the laboratory determined that the increases in

UGT1A expression observed during pregnancy were also maintained in the Tg-*UGT1*-PXRnull mothers.

Characterization of the Tg-*UGT1*-PXRnull line revealed an interesting phenomenon in liver samples, however. Basal expression of *UGT1* genes was significantly higher in Tg-*UGT1*-PXRnull compared to Tg-*UGT1*. Because PXR has been implicated in cross-regulation of other nuclear receptors, including CAR and AhR, I hypothesized that these differences were due to dysregulation of the CAR or AhR signaling pathways in the absence of PXR. Regulatory studies using CAR and AhR ligands in Tg-*UGT1* and Tg-*UGT1*-PXRnull mice were undertaken to determine the role of PXR in the expression and induction of the *UGT1* locus.

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CHAPTER 2

Induction of UGT1A1 by the Nuclear Receptor PPAR α

Introduction

The Peroxisome Proliferators-Activated Receptor α (PPAR α) belongs to the adopted orphan class of nuclear receptor and is linked to regulation of genes involved in lipid and bile acid metabolism. Its endogenous ligands include fatty acids, oxidized phospholipids, and eicosanoids (Delerive et al., 2000; Willson et al., 2000), but the receptor is pharmaceutically relevant because it can be activated by a class of synthetic anti-hyperlipidemic drugs known as fibrates (gemfibrozil, ciprofibrate, fenofibrate) (Willson et al., 2000). Fibrates exert their actions through PPAR α , which binds as a PPAR α / RXR heterodimer to recognition sequences in the regulatory regions of genes involved in fatty acid oxidation. These drugs as well as endogenous PPAR α agonists are excreted as glucuronides, implying a role for UGTs in their detoxification (Liu et al., 1991; Wheelan et al., 1999). Additionally, fibrate drug treatment in humans and laboratory animals has been shown to increase glucuronidation and UGT expression (Hautanen et al., 1992; Mounie et al., 1988; Richert et al., 2003; Senekeo-Effenberger et al., 2007; Turgeon et al., 2003), thereby stimulating a feedback loop for the drug's clearance. An important consequence of UGT induction by fibrates, however, is the increased glucuronidation of a host of endogenous and xenobiotic UGT substrates. In rats, increased bilirubin glucuronidation and a concomitant increase in liver UGT1A1 protein levels was observed after treatment with fibrate drugs (Mounie et al., 1988; Viollon-Abadie et al., 2000; Jemnitz et al., 2000); in humans treated with gemfibrozil, elevated plasma androstane-3 α , 17 β -diol glucuronide was detected (Hautanen et al., 1992). By determining the extent of the regulatory effect of PPAR α on the human UGTs, it may

be possible to predict negative consequences of fibrate treatment in patients with impaired UGT function or in patients under polypharmaceutical treatment.

In recent years, human UGT1A9 and UGT2B4 have been identified as targets for PPAR α , and DR1 response elements in the promoters of these genes have been characterized (Barbier et al., 2003b; Barbier et al., 2003a). As UGT1A9 and 2B4 can glucuronidate fibrates, polyunsaturated fatty acids, and other endogenous PPAR α ligands (Turgeon et al., 2003; Barbier et al., 2003b; Barbier et al., 2003a), these were logical putative PPAR α target genes. The observed increased bilirubin and steroid glucuronide formation after fibrate treatment, however, suggests UGT1A1 and other UGTs as PPAR α targets as well. My laboratory undertook experiments using Tg-*UGT1* mice to determine PPAR α -mediated induction of the human *UGT1* genes in vivo. Liver *UGT1A1*, *1A3*, *1A4*, and *1A6* were induced following treatment with the fibrate WY-14643 (WY) in these mice, with UGT1A1 transcript demonstrating a 500-fold increase. Induction of UGT1A1, 1A4, and 1A6 protein was also observed in liver, and these proteins were also significantly induced in the small intestine and colon.

I undertook studies to understand the molecular regulation of the UGT1A1 gene by PPAR α using ex vivo Tg-*UGT1* hepatocyte cultures as well as a human hepatoma-derived immortalized cell line, HepG2. My results demonstrate that UGT1A1 is directly regulated by PPAR α through a DR1 element located in the PBREM region of the UGT1A1 promoter/enhancer.

Materials and Methods

Reagents – Pirinixic acid (4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid), commonly known as WY-14643, and DMSO were purchased from Sigma-Aldrich (St. Louis, MO). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was obtained from Wellington Laboratories (Guelph, Ontario, Canada). Human-specific UGT1A1 and UGT1A4 antibodies (Chen et al., 2005) were kindly provided by Dr. Joseph Ritter from Virginia Commonwealth University, Richmond, Virginia. A rat CYP4A1 antibody originally generated as described previously (Okita and Okita, 1992) was donated to us by Dr. Eric Johnson from the Scripps Research Institute, La Jolla, CA. The rat CYP4A1 is most homologous to mouse Cyp4a10, but in experiments outlined here the mouse protein(s) identified with the rat CYP4A1 antibody are referred to as Cyp4a. Human HepG2 cells that express a stably integrated hPPAR α cDNA were provided by Dr. Eric Johnson (Hsu et al., 2001).

Isolation and Treatment of Mouse Transgenic Primary Hepatocytes – Primary hepatocytes were isolated from 8- to 12-week old male mice. Mice were anesthetized by isoflurane inhalation. The portal vein was cannulated and perfused with Hank's balanced salt solution (Ca²⁺-free and Mg²⁺-free) containing 0.1 mM EGTA and 10 mM Hepes, pH 7.4, for 5 min at the rate of 7 mL/min. Immediately after the start of perfusion, the anterior vena cava was cut to allow continuous flow to proceed out of the liver. The perfusate was then changed to a solution containing 20 μ g/mL Liberase Blendzymes (Roche Applied Science) dissolved in Hank's balanced salt solution (with Ca²⁺ and Mg²⁺), and the perfusion was continued for another 5 min. The liver was removed, and the hepatocytes were isolated by mechanical dissection followed by

filtration through a sterile 70- μ m filter. The cells were immediately collected by centrifugation at 50 x g for 30 sec and were washed in Dulbecco's modified Eagle's tissue culture media. Cell viability was examined by trypan blue exclusion, and experiments were conducted only if viability exceeded 90%. The hepatocytes were then cultured in 6-well collagen-treated plates (Discovery Labware, Bedford, MA) in 3 mL of Dulbecco's modified Eagle's medium containing penicillin/streptomycin and supplemented with 10% fetal bovine serum. Three hours after plating, the medium was replaced. 24 hours after seeding, the hepatocytes were treated with DMSO or either 50 μ M or 100 μ M WY for a total of 48 hours, replacing the media and chemical after 24 hours.

Protein Analysis by Western Blot – To collect whole cell lysates, hepatocytes were lysed in a buffer containing 0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.25% deoxycholic acid, and 1% Nonidet P-40 with a complement of protease and phosphatase mixture inhibitors (Sigma). After incubation of this mixture for 30 min on ice, the solubilized lysate was centrifuged for 20 min in a refrigerated Eppendorf centrifuge at 16,000 x g. The supernatant was collected and used directly for Western blot studies. All Western blots were performed using NuPAGE BisTris-polyacrylamide gels as outlined by the supplier (Invitrogen). Protein was heated at 70 °C for 10 min in loading buffer and resolved in 4–12% BisTris gels under denaturing conditions (50 mM MOPS, 50 mM Tris base, pH 7.7, 0.1% SDS, 1 mM EDTA) prior to transferring the proteins to polyvinylidene difluoride membrane using a semidry transfer system (Novex). The membrane was blocked with 5% nonfat dry milk in

washing buffer [10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and 0.05% Tween 20 (Tris-buffered saline)] for 1 h at room temperature, followed by 5 x 10 minutes washes in washing buffer and overnight incubation with primary antibodies (mouse anti-human UGT1A1, or UGT1A4, or rabbit anti-rat Cyp4a1) diluted 1:5000 in Tris-buffered saline at 4 °C. Membranes were washed and exposed to horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology Inc., Danvers, MA) for 1 h at room temperature. After additional washes in washing buffer, the horseradish peroxidase-conjugated protein was detected with the Western Lightning™ Chemiluminescent detection system (Perkin-Elmer) and a ChemiDoc XRS chemiluminescent camera (BioRad).

RNA isolation and Analysis of Gene Expression Using RT-PCR – For RNA isolation from hepatocytes, 500 µL of acidic phenol-guanidinium isothiocyanate solution (TRIzol, Invitrogen) was added to each well and total RNA was extracted according the manufacturer's protocol. cDNA was synthesized from 1 µg of total RNA (isolated as described) using the iScript cDNA synthesis kit (BioRad) method. For each 20 µL PCR reaction, 1 µL of the reverse transcription reaction was used as template, along with 200 nM of forward and reverse mouse β-actin primers, 300 nM of UGT1A-specific primers (Strassburg et al., 1998;Strassburg et al., 1999), 5 mM dNTP mix, and 1 unit of Taq (Denville) in proprietary buffer. PCR was performed as described (Chen et al., 2005). 10 µL of each reaction was visualized on a 1.8% agarose gel containing 1 µg/mL ethidium bromide. Primer sequences are listed in Table 2-1. Quantitative real-time PCR was performed on a Stratagene MX4000

Table 2-1. UGT1A Oligonucleotide Sequences Used for RT-PCR. UGT1A1Q, UGT1A3Q, UGT1A4Q, and UGT1A6Q were primers designed for and used in Quantitative Real-time PCR.

	<i>Forward primer (5'-3')</i>	<i>Reverse primer (5'-3')</i>
<i>UGT1A1</i>	aacaaggagctcatggcctcc	gttcgcaagattcgatggtcg
<i>UGT1A3</i>	tggtgaacaatatgtctttggtcta	gttcgcaagattcgatggtcg
<i>UGT1A4</i>	gaaggaatttgatcgcgttac	gttcgcaagattcgatggtcg
<i>UGT1A6</i>	tcttggtgagtatattgggcc	gttcgcaagattcgatggtcg
<i>UGT1A1Q</i>	aacaaggagctcatggcctcc	gttcgcaagattcgatggtcg
<i>UGT1A3Q</i>	cagtggaggataattctcagtc	ccatgttctccagaacgatta
<i>UGT1A4Q</i>	acgctggggctacactcaagg	tcattaatgcagtagctccacaaa
<i>UGT1A6Q</i>	ctttcacagacccagccttac	tatccacatctctcttgaggacag
<i>mβ-actin</i>	ggcggcaccaccatgtaccct	aggggccggactcgtcatact
<i>mCPH</i>	atggtcaacccccaccgtgt	ttcttgctgtctttggaactttgtc

Multiplex QPCR. 1 μ L of iScript cDNA product was used in 20 μ L PCR reactions with Mesa Green qPCR MasterMix (Eurogentec, San Diego CA), and each sample was run in triplicate. Quantitative PCR analysis for UGT1A1, UGT1A3, UGT1A4, and UGT1A6, mRNA levels were determined with the sense and antisense primers listed in Table 2-1. For UGT1A1 and UGT1A6, each PCR reaction was carried out at 95°C for 10 min, 95°C for 30 s, and 63°C for 60 s, followed by 72°C for 45 s, processed through 40 cycles. Amplification was followed by DNA melt at 95°C for 1 min and a 41-cycle dissociation curve starting at 55°C and ramping 1° every 30 s. For UGT1A3, each PCR reaction was carried out at 95°C for 10 min, 95°C for 30 s, and 58°C for 60 s, followed by 72°C for 45 s, processed through 40 cycles. To determine the quantitation of gene expression, the comparative threshold cycle method (ΔC_T) was used (Livak and Schmittgen, 2001). Mouse carboxypeptidase-H (mCPH), a housekeeping gene, was used to control for variations in mRNA quantity and quality; subtraction of the mCPH C_t values from the C_t of the *UGT* gene of interest yielded the ΔC_t value.

PPAR α Isolation and Expression – PPAR α cDNA was isolated from human liver RNA using RT-PCR with 5'-atggtggacacggaaagc-3' as a forward primer and 5'-tcagtacatgtccctg-3' as a reverse primer. Two products were isolated, reflecting the two splice variants of PPAR α found in human liver (Sapone et al., 2000); only the higher molecular weight band representing the intact, fully-functional receptor was isolated and cloned into pcR-2.1 TOPO (Invitrogen) according to the manufacturer's protocol. NheI and KpnI restriction sites incorporated into the forward and reverse

primers facilitated excision of the cDNA from the TOPO vector and subcloning into a pcDNA 3.1 (-) expression vector. Transfection of the hPPAR α -pcDNA expression vector into HepG2 cells was performed using LipofectAMINE 2000 (Invitrogen) and 200 ng of plasmid DNA. 24 hours after transfection, the cells were treated with DMSO or 100 μ M WY dissolved in DMSO for 48 hours, replacing the medium and chemical after 24 hours. RNA was isolated as described above and RT-PCR was performed using primers specific for UGT1A1, UGT1A4, and m β -actin.

UGT1A1 Promoter Cloning and Expression – Human liver DNA was used as a template for PCR to isolate enhancer DNA fragments on the *UGT1A1* promoter. Oligonucleotide primers were designed to incorporate restriction sites onto the 5' (SacI) or 3' (XhoI) ends to facilitate cloning into pGL3 basic or SV40-pGL3 promoter vectors. Primers are listed in Table 2-2. PCR products were first cloned into the pcR-2.1 TOPO vector (Invitrogen, La Jolla, CA) according the manufacturer's protocol. The UGT1A1 promoter fragments were excised from the pcR-2.1 TOPO vector by SacI and XhoI digest and were subcloned into the appropriate pGL3 reporter vector. For analysis of functional DR1 elements, complimentary oligonucleotides encoding three copies of the UGT1A1 (5'-gcccaAGGGTAGAGTTCAgtgt-3') DR1 sequence were synthesized, annealed, and cloned into the SV40pGL3 expression vector. All transfection experiments were conducted in triplicate using HepG2 cells and HepG2 cells that carry an integrated PPAR α cDNA (PPAR α -HepG2). HepG2 and PPAR α -HepG2 cells were cultured to ~80% confluence in 12-well plates and transfections were performed using 200 ng of

Table 2-2. UGT1A1 Promoter Oligonucleotides. Forward and reverse primer pairs used to clone the UGT1A1 promoter constructs used in this study are listed. The E3mut primers are reverse complementary and contain mutated residues in **bold** and underlined.

<i>Construct</i>	<i>Oligonucleotide sequence (5' → 3')</i>	<i>Location</i>
R1	F: aaagaagagaact R: gggaatgatccttc	-11001 -8125
R2	F: ttgcttgctgcc R: aacatggctggtt	-8537 -4636
R3	F: gattcattggaccac R: gttcgccctc	-5481 -7
PR	F: tcagacaaaaggaa R: gttcgccctc	-3714 -7
E1	F: tcagacaaaaggaa R: aaccactactaagc	-3714 -2068
E2	F: tatcccagagttt R: aaccactactaagc	-3126 -2068
E3	F: ttttgacactgga R: ctcattcctcctct	-3526 -3143
E3mut	F: cttggttaag ac cgcaatgaac R: gttcattg cg <u>gt</u> cttaccaag	-3286 -3267
E4	F: taacggttcataaa R: ctactatgactg	-3430 -3272

plasmid DNA that had been isolated within 3 days prior to transfection along with 5 ng of phRL-SV40, a plasmid encoding the *Renilla reniformis* luciferase gene under a constitutive promoter, using LipofectAMINE 2000 reagent according to the manufacturer's protocol (Invitrogen). After 24 h, the cells were treated for 48 h with 100 μ M WY-14643 dissolved in DMSO or vehicle alone. Dual luciferase assays were conducted according to the manufacturer's instructions (Promega). While the cells were still attached, 200 μ l of Passive Lysis Buffer (Promega) was added, and 20 μ l of lysate was used for dual luciferase analysis using an LMax II³⁸⁴ luminometer. Firefly luciferase values were normalized to *Renilla reniformis* luciferase and protein concentration and reported as -fold increase over vehicle-treated cells.

Results

Primary hepatocytes isolated from Tg-*UGT1* mice were treated with solvent (DMSO), 50 μ M WY, 100 μ M WY, or 10 nM TCDD as a positive control for 48 hours, and UGT1A transcript levels were quantified by Quantitative RT-PCR. A 2-fold increase in UGT1A1 transcript was observed in cells treated with 50 μ M WY, while a statistically significant 3.7-fold increase was observed with 100 μ M treatment (figure 2-1). These were relatively low compared to the potent induction by TCDD, however, even in vivo, induction by WY is appreciably lower than by TCDD. Despite the high degree of homology among the UGT sequences, isoform-specific oligonucleotide primers generated previously in the laboratory (Strassburg et al., 1997; Chen S et al., 2005) were used to amplify UGT1A1 cDNA specifically.

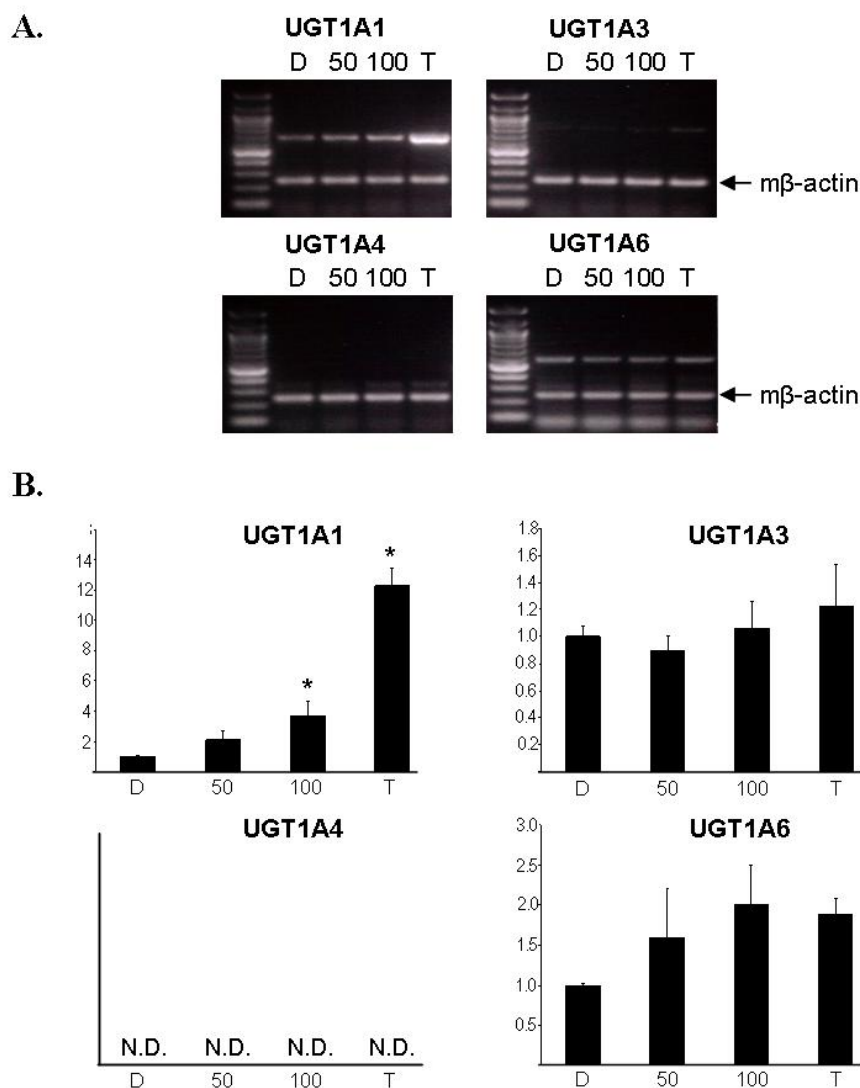


Figure 2-1. Induction of UGT1 Transcripts by PPAR α in Tg-UGT1 Hepatocytes. Hepatocytes isolated from livers of Tg-UGT1 mice were plated and treated with DMSO (D), 50 μ M WY-14643 (50), 100 μ M WY-14643 (100), or 10 nM TCDD (T). Total RNA was collected. 1 μ g total RNA was used for each RT reaction. **A)** RT-PCR was performed at 30 cycles using isoform-specific primers with m β -actin as an internal control. **B)** Quantitative RT-PCR using isoform-specific primers was run using CPH as an internal control. Values are normalized for each isoform to the average of the DMSO-treated samples. N.D. = Not Detected, for samples generating a Ct value above 35. Statistically significant change over DMSO as determined by the Student's *t*-test is indicated (*, $p \leq 0.05$; **, $p \leq 0.005$).

Isoform-specific amplification of mRNA from these hepatocytes also demonstrated that while UGT1A1 appears to be moderately upregulated by WY, UGT1A3, 1A4, and 1A6 show little to no induction, and UGT1A4 was undetectable at 35 PCR cycles. Analysis of whole cell lysate showed increases in UGT1A1 and Cyp4a protein levels in response to WY, while UGT1A4 was not detected (Figure 2-2).

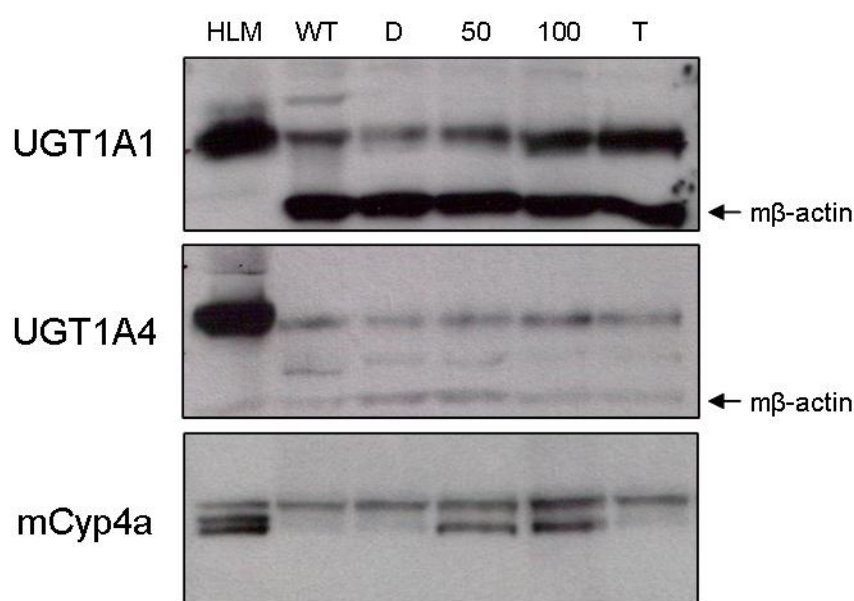


Figure 2-2. Induction of UGT1 Protein by PPAR α in Tg-UGT1 Hepatocytes. Hepatocytes isolated from livers of Tg-UGT1 mice were plated and treated with DMSO (D), 50 μ M WY-14643 (50), 100 μ M WY-14643 (100), or 10 nM TCDD (T). Whole cell lysates were collected. Each sample represents a pool of three individual wells. 20 μ g total protein was applied to each well. Membranes were probed with antibodies specific for human UGT1A1, human UGT1A4, or mouse Cyp4a. Each blot was run in duplicate and was probed with an antibody directed against mouse Actin; corresponding blots were overlaid.

These findings contrast with *in vivo* work performed concurrently in the laboratory which demonstrated that UGT1A3 transcript is highly upregulated in the livers of Tg-UGT1 mice treated with WY, and UGT1A4 transcript is also increased

upon WY treatment, though not as greatly as UGT1A1 or 1A3 (Senekeo-Effenberger et al., 2007). The decrease in UGT1A3 and 1A4 expression or inducibility was later determined to occur within 8 hours of hepatocyte plating, and mirrors the expression profiles observed in the immortalized hepatoma cell line HepG2 (data not shown).

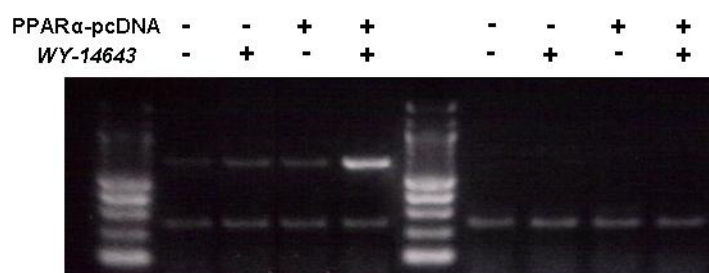


Figure 2-3. Expression of PPAR α in HepG2 Cells Restores WY Induction of UGT1A1 but not UGT1A4. HepG2 cells were transfected with empty pcDNA 3.1 or pcDNA 3.1 expressing PPAR α cDNA. Cells were treated with either DMSO or 100 μ M WY for 48 hours. RNA was collected and RT-PCR was run using isoform-specific primers and mouse β -actin as an internal control for 28 cycles.

Although UGT1A1 is expressed in HepG2 cells and was shown to be inducible in these cells by the AhR (Yueh et al., 2003), very little induction was observed by the prototypical PPAR α ligand WY (figure 2-3). Indeed, PPAR α control genes such as Cyp4a and HMG-CoA Reductase are not inducible in HepG2 cells due to very low levels of PPAR α protein (Lawrence et al., 2001; Hsu et al., 2001). Thus, studies to clone the human PPAR α from human liver cDNA into a pcDNA3.1 expression vector for transfection into HepG2 cells were undertaken. Two splice forms of PPAR α are present in human liver; only the longer form is fully functional and this was the variant that was isolated and cloned into the expression vector. Transient transfection of the PPAR α -pcDNA plasmid construct into HepG2 cells

allowed for induction of UGT1A1 (figure 2-3), although UGT1A4 was still not inducible by WY.

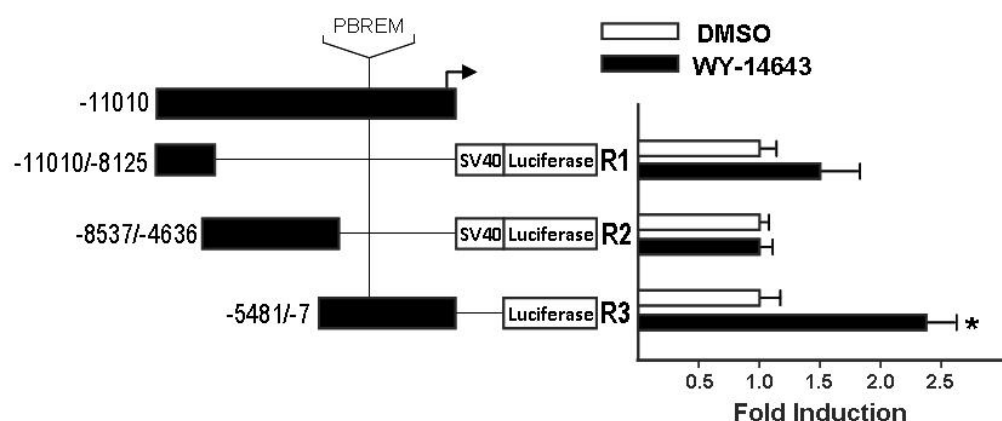


Figure 2-4. Identification of WY-Responsive Portions of the *UGT1A1* Promoter. Three overlapping constructs encompassing the 11 kb of regulatory sequence extending in the 5' direction from the *UGT1A1* gene were cloned into SV40-pGL3 luciferase (R1, R2) or pGL3-basic luciferase (R3) vectors which were transfected into hPPAR α -HepG2 cells (Hsu et al., 2001). After transfections, the cells were treated with WY-14643 (100 μ M) for 48 h before analysis of luciferase activity. Fold induction was calculated by dividing the activity generated from WY-1643 treatment with the activity from the same set of transfected cells treated with DMSO. * indicates statistical significance as calculated by the Student's *t* test ($p < 0.05$).

To further identify the molecular mechanism for PPAR α induction of UGT1A1, promoter constructs of varying lengths fused with a luciferase reporter gene were created. Approximately 11 kilobases of regulatory sequence are present between the 5' end of the exon 1 of UGT1A2p and the UGT1A1 transcriptional start site. Three overlapping constructs encompassing the entire 11 kb regulatory region were generated. Transient transfection of these constructs into HepG2 cells stably expressing PPAR α (hPPAR α -HepG2) (Hsu et al., 2001) localized PPAR α response to

the 5.5 kb region (R3) proximal to the UGT1A1 gene (figure 2-4), although a slight non-statistically significant response was detected with the R1 construct. Within this R3 region exists an important regulatory element for nuclear receptor control of the UGT1A1 gene, the PBREM (Phenobarbital Responsive Element). Initially identified as the region conferring responsiveness to CAR (Sugatani et al., 2001; Xie et al., 2003), this region 3.2-3.5 kb upstream from the UGT1A1 gene also contains the primary response elements for PXR (Xie et al., 2003), AhR (Yueh et al., 2003), and Glucocorticoid Receptor (Sugatani et al., 2005). Sequence analysis of this region revealed a putative DR1 site in the 5' end of the PBREM (figure 2-6). A 3.7 kb promoter construct previously shown to contain the PBREM (Yueh et al., 2003) was analyzed for WY response (figure 2-5); WY induced luciferase activity in hPPAR α -HepG2 but there was no induction in HepG2 cells. Enhancer constructs containing the PBREM (E1 and E3) were WY-responsive, while deletion of the PBREM (E2) resulted in a loss of inducibility, confirming the presence of a PPAR α -responsive element (PPRE) within the PBREM. Mutational analysis of the DR1 site was performed at residues determined to be essential for activation by the PPAR α /RXR heterodimer (Juge-Aubry et al., 1997; Castelein et al., 1997). Mutation of these residues abolished luciferase activity. To further confirm activation of this DR1 element by PPAR α , an oligonucleotide concatamer containing 3 repeats of the putative UGT1A1 DR1 element was fused with a pGL3-promoter luciferase reporter (figure 2-6). Transfection of this construct into either HepG2 or hPPAR α -HepG2 cells and treatment with WY resulted in a significant increase in luciferase activity in only the hPPAR α -HepG2 cell line.

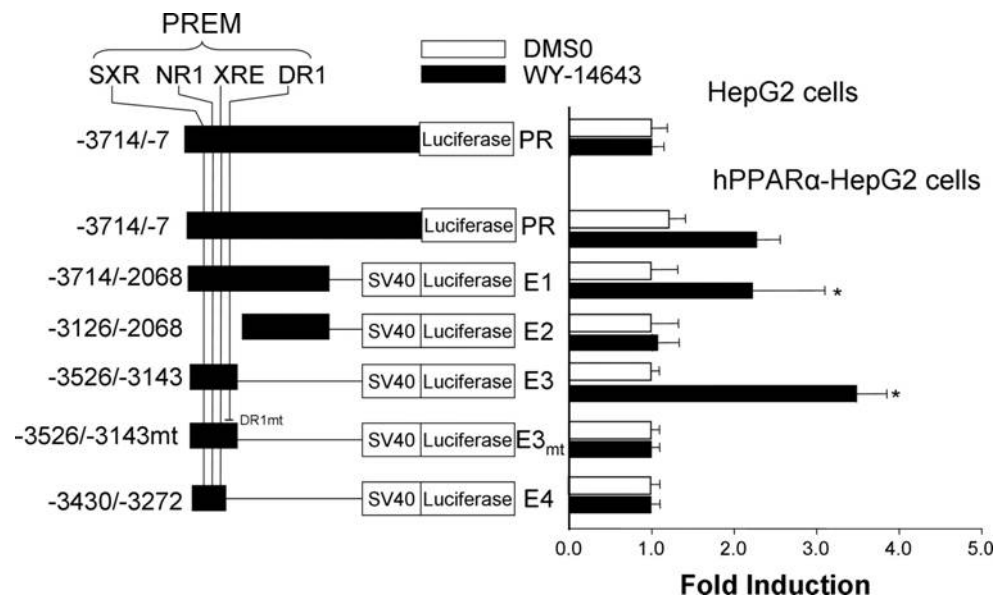


Figure 2-5. Activation of the *UGT1A1* Promoter in Response to WY-14643. A portion of the *UGT1A1* gene containing the promoter and -3712 bases (-3712/-7 PR) of the regulatory region previously shown to be responsive to activated PXR, CAR, and the Ah receptor (Xie et al., 2003; Yueh et al., 2003) was transiently transfected into either HepG2 cells or hPPARα-HepG2 cells (Hsu et al., 2001). Additional enhancer constructs containing the PREM region (E1 and E3) as well as constructs that were deficient the DR1 element (E2 and E4) were subcloned into the pGL3-promoter vector. Each of these plasmids was transfected into PPAR-HepG2 cells. After transfections, the cells were treated with WY-14643 (100 μM) for 48 h before analysis of luciferase activity. Fold induction was calculated by dividing the activity generated from WY-14643 treatment with the activity from the same set of transfected cells treated with DMSO. * indicates statistical significance as calculated by the Student's *t* test ($p < 0.05$).

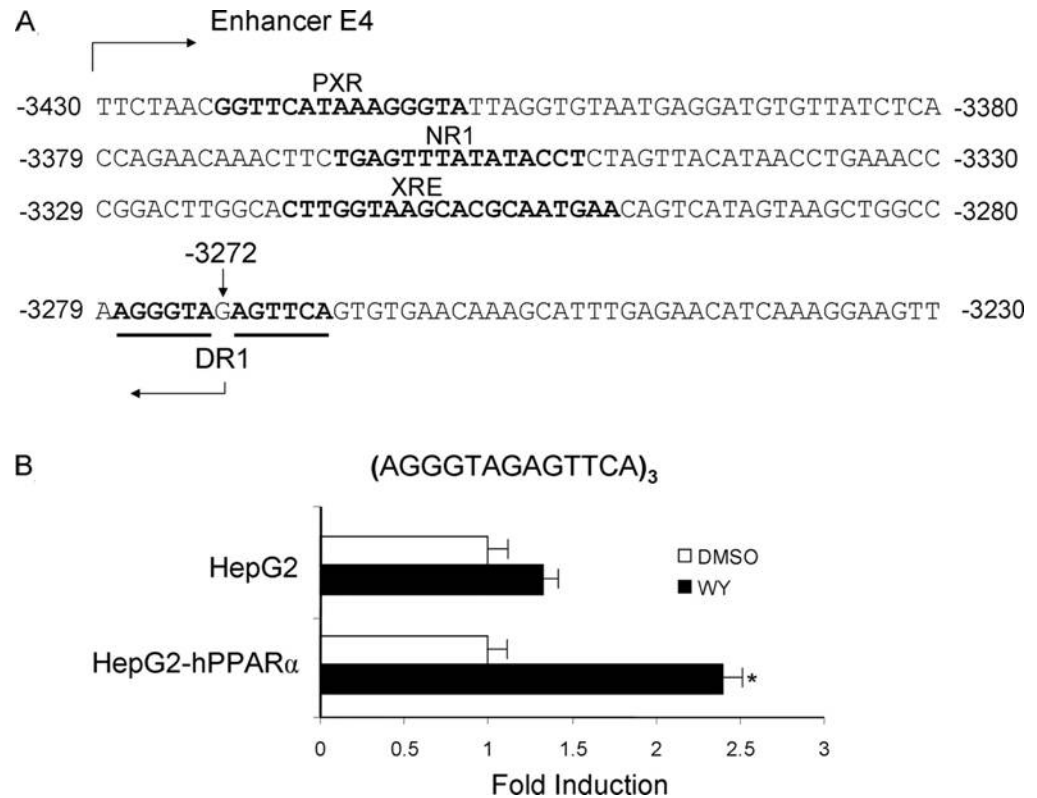


Figure 2-6. DNA Sequence of the PBREM Flanking the *UGT1A1* Gene. **A)** DNA sequence of the *UGT1A1* gene from bases -3430 to -3230 is shown. Included in this sequence are the DNA binding regions for the PXR, CAR (NR1), Ah receptor (XRE), and PPAR (DR1), as indicated by the sequence in bold. Enhancer element E4 spans the DNA sequence from -3420 to -3275. **B)** The *UGT1A1* DR1 sequence containing three copies of the DR1 (agggttagagttca) was cloned into the pGL3 reporter plasmid and was transfected into HepG2 and hPPAR α -HepG2 cells. The cells were treated with vehicle (DMSO) or WY-14643 (100 μ M) for 48 h followed by analysis of luciferase activity.

Discussion

In vivo experiments using Tg-*UGT1* mice demonstrated that the UGT1As are highly upregulated in response to fibrate treatment, and UGT1A induction was observed in several organs, including the liver, small intestine, and kidney (Senekeo-Effenberger et al., 2007). Notably, UGT1A3 and 1A1 were highly induced in the liver, with observable but much lower induction of 1A4 and 1A6. Clearly, fibrate treatment can have a dynamic effect on the detoxification process: UGT1A3 is the primary enzyme responsible for glucuronidation of certain bile acids and UGT1A1 is the sole enzyme that glucuronidates bilirubin. Disruption of the clearance of these important endogenous compounds can lead to disease phenotypes such as liver cholestasis or hyperbilirubinemia (Verreault et al., 2006). In addition to inducing the clearance of endogenous compounds susceptible to glucuronidation, clearance of other therapeutic drugs may be affected by fibrate therapy. As many pharmaceutical drugs require plasma concentrations to remain within a specific range to maximize efficacy, combination therapy with fibrates may cause increased clearance and a subsequent reduction in medical benefit from those drugs. Fibrates are used to treat a variety of medical conditions, including type 2 diabetes, hyperlipidemia, insulin resistance, and coronary artery disease (Li et al., 2000; Li and Palinski, 2006; Rubins et al., 2003), and their use is increasingly wide-spread. Thus it is important to understand and identify the molecular effect of PPAR α on drug metabolism. Given the importance of glucuronidation on the clearance of many pharmaceutical drugs, identification of PPAR α -responsive elements on *UGT1* genes will provide conclusive

evidence for the ability of PPAR α to directly regulate these important contributors toward endogenous and xenobiotic metabolism.

In hepatocyte cultures isolated from the livers of Tg-*UGT1* mice, the PPAR α ligand WY induced UGT1A1 and UGT1A6, but did not induce UGT1A3 or 1A4 (figures 2-1, 2-2). This was a surprising finding, given that UGT1A3 was most highly upregulated in the livers in vivo (Senekeo-Effenberger et al., 2007). Additional unpublished studies in the laboratory revealed that these mouse primary hepatocytes demonstrated a rapid downregulation and loss of inducibility of UGT1A3 and 1A4 within the first 8 hours after plating. UGT1A1 and 1A6 retained inducibility, though basal expression and induction was of a much lower magnitude than in vivo. This result differs significantly from human hepatocyte cultures, in which expression and induction of UGT1A1, 1A3, 1A4, and 1A6 are intact (Senekeo-Effenberger et al., 2007). Interestingly, HepG2 cells display a pattern of expression and induction of the UGTs similar to that observed in the Tg-*UGT1* hepatocytes, with little to no expression of UGT1A3 or 1A4. It has recently been suggested that the loss of expression or induction of many drug metabolism genes in the HepG2 line may be attributed to microtubule disarray during the post-attachment morphological changes that occur after plating, and this disruption can prevent nuclear receptors from translocating to the nucleus, effectively shutting down basal and inducible expression of certain drug metabolism genes (Horiuchi et al., 2009; Dvorak et al., 2005). Thus, the implementation of new culture techniques or chemical treatments to prevent microtubule disarray may allow for use of HepG2 or Tg-*UGT1* hepatocyte cultures in the study of UGT1A3, 1A4, and other drug metabolism genes.

In HepG2 cells, although protein levels of many nuclear receptors are very low, it appears that overexpression can be sufficient to reconstitute these signal transduction pathways; transient transfection of these cells with PXR or CAR expression vectors followed by treatment with the appropriate nuclear receptor agonist has been shown to induce UGT1A1 (Xie W et al., 2003; Sugatani J et al., 2004). In this study, PPAR α was cloned from human liver cDNA into a pcDNA expression vector for transient transfection into HepG2 cells. In human liver, two splice forms of PPAR α are present: the full-length transcript and a truncated non-functional transcript that lacks exon 6 (Palmer et al., 1998). Only the full-length variant was isolated and used in this study. Transient transfection of the human PPAR α into HepG2 cells followed by treatment with WY resulted in robust induction of UGT1A1 transcript (figure 2-3). Curiously, reconstituting the PPAR α signaling pathway was not sufficient to restore UGT1A4 induction, indicating that the missing factor or factors required for basal expression in these cells may also be required for induction of the gene.

Development of a HepG2 cell line in which human PPAR α cDNA is stably integrated into the genome (hPPAR α -HepG2) (Hsu et al., 2001) provided a cell culture system in which to examine PPAR α interaction with the UGT1A1 promoter and enhancer sequence. UGT1A1 promoter constructs were cloned and fused with a luciferase reporter gene in pGL3 basic or pGL3 promoter vectors. Of the 11 kb of UGT1A1 regulatory sequence, PPAR α activation was first localized to the proximal 5.5 kb (figure 2-4). Within that 5kb sequence, a DR1 element that correlated well with known PPRES was identified in the PBREM at -3.3 kb (figure 2-6). Serial

deletion of PBREM constructs revealed that PPAR α response was restricted to a small region that included the putative PPRE (figure 2-5). Mutation of the putPPRE and creation of a 3x concatenated response element confirmed that PPAR α acts on UGT1A1 through this 20 bp element (figure 2-6).

UGT1A1, like many of the *UGT1* genes, is under control of many nuclear receptors, including PXR, CAR, AhR, GR, and PPAR α . Its regulatory sequence, however, is unique in that the response elements for all of these receptors are tightly clustered in a *trans*-acting response module. The physiological implications of such closely-spaced regulatory elements is unclear; activation of multiple signal transduction pathways may allow for a combinatorial effect, resulting in increased or more rapid recruitment of transcriptional machinery, or steric limitations may result in competition for DNA binding. What is clear is that UGT1A1, an enzyme that is critical for human health, is tightly controlled by the nuclear receptors, allowing the gene to be activated in the presence of xenobiotics such as the fibrates. By understanding the extent and control of PPAR α -mediated induction, it is possible to predict alterations in drug metabolism profiles and subsequent changes in the clearance of drugs taken in combination with fibrate therapy.

Acknowledgements

The identification of the PPAR α -responsive element on the *UGT1A1* promoter, figures 2-5 and 2-6, are reprinted from *Drug Metabolism and Disposition* 2007, Senekeo-Effenberger K, Chen S, **Brace-Sinnokrak E**, Yueh MF, Bonzo JA,

Argikar U, Kaeding J, Trottier J, Remmel RP, RitterJK, Barbier O, Tukey RH. Expression of the human *UGT1* locus in transgenic mice by Wy-14643 and implications on drug metabolism through PPAR α activation. **35** (3):419-27. I identified the PPRE through promoter-transfection studies, constructed the concatenated PPRE-reporter, and performed the luciferase experiments.

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CHAPTER 3

Induction of UGT1A4 by the Nuclear Receptors

Introduction

The development of a Tg-*UGT1* mouse in 2005 (Chen S et al., 2005) created the opportunity to investigate regulation of the human *UGT1* gene locus by conserved nuclear receptor signaling pathways. Induction of *UGT1* expression by PXR and AhR ligands was observed in these mice; interestingly, all of the *UGT1* genes were capable of induction by PXR and AhR, either in the liver or intestine (Chen S et al., 2005). Robust induction of UGT1A1 and 1A3 in the liver and 1A1 and 1A4 in the small intestine was observed when these mice were treated with the PPAR α agonist WY-14643 (Senekeo-Effenberger et al., 2007). The *UGT1* genes are clearly responsive to a complement of nuclear receptors, however, in the ubiquitous immortalized human hepatocyte cell line HepG2, minimal expression or induction of UGT1A3, 1A4, or 1A7-10 is observed after treatment with PXR, CAR, AhR, or PPAR α agonists. Additionally, hepatocytes from Tg-*UGT1* mice lose basal or inducible expression of UGT1A3 and 1A4 within hours of plating. These data suggest that basal regulatory factors that are required for normal expression of UGT1A3 and 1A4 are lost in vitro; these may be circulating humoral factors acting in a paracrine or endocrine manner, endogenous ligands, or a disruption of signaling pathways that are critical for the expression of these two genes. Microtubule disruption, as suggested in the previous chapter, has been most recently suggested as the factor mediating down-regulation of many drug metabolism genes in HepG2 cells (Horiuchi et al., 2009), though UGT1A3 or 1A4 were not specifically examined in this study.

UGT1A3 and 1A4 are two closely related enzymes, sharing over 93% amino acid identity, and have similar tissue expression profiles (Chen et al., 2005; Tukey and Strassburg, 2000). They are of particular interest to human health and drug metabolism because they are uniquely capable of forming quaternary ammonium-linked glucuronides from substrates possessing a tertiary amine chemical moiety (Green and Tephly, 1998; Porter et al., 1975). These substrates include a host of pharmaceutical drugs, including antihistamines, tricyclic antidepressants, and antipsychotics (Chaudhuri et al., 1976; Luo et al., 1991b; Luo et al., 1991a; Luo et al., 1995; Lehman et al., 1983). Glucuronidation of most of these drugs by UGT1A3 appears to be less efficient than by UGT1A4, which efficiently glucuronidates many compounds in these three classes and is the primary enzyme responsible for the clearance of several of these drugs (Green and Tephly, 1998). Additionally, UGT1A4 glucuronidates endogenous progestins and certain androgens with high rates and with high efficiency (Green and Tephly, 1996). Despite the high degree of homology between UGT1A3 and 1A4, the substrate specificities are divergent: although both are capable of forming quaternary ammonium-linked glucuronides, UGT1A3 targets aromatic hydroxyl and carboxylic acid moieties while aliphatic hydroxyl groups, such as those found on progestins and androgens, are the site of glucuronide attachment for UGT1A4.

Laboratory rodents do not possess the ability to glucuronidate tertiary amine compounds, due to a mutation in the rodent *UGT1A4* gene that results in a premature stop codon and leads to a non-functional truncated UGT1A4 protein. As UGT1A4 is an important enzyme in the clearance of several common clinically used drugs, this

represents a critical deficiency when using rat or mouse models for preclinical investigations into drug safety or drug-drug interactions. The development of the Tg-*UGT1* mouse model has allowed for in vivo characterization of the regulatory pathways that govern its expression, and the Tukey laboratory has demonstrated that UGT1A4 is upregulated by PXR, AhR, and PPAR α agonists in the liver and intestines (Chen S et al., 2005; Senekeo-Effenberger et al., 2007). Thus far, however, owing to the lack of 1A4 expression in hepatocyte or HepG2 cell, and the apparent dysfunction of several relevant nuclear receptor pathways in these cells, a further characterization of the regulatory elements responsible for this upregulation has not been possible. In this study, through use of a non-hepatoma cell line that exhibits functional PXR, PPAR, and AhR signal transduction pathways, PPAR, AhR, and putative PXR response elements are identified within the UGT1A4 regulatory sequence.

Materials and Methods

Reagents – Pregnenolone-16 α -carbonitrile (PCN), Pirinixic acid (4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid) (WY-14643), and DMSO were purchased from Sigma-Aldrich (St. Louis, MO). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was obtained from Wellington Laboratories (Guelph, Ontario, Canada).

Maintenance and Treatment of LS180 Cells – LS180 cells were obtained from ATCC and were cultured in Minimal Essential Medium supplemented with 10% Fetal Bovine Serum, 1% non-essential amino acids, and 2mM glutamine. Cells were

grown in 6-well plates to ~70% confluence and were then treated with either DMSO or 10 nM TCDD, 100 μ M WY-14643, or 10 μ M Rifampicin dissolved in DMSO. After 24 hours, medium was replaced with fresh medium supplemented with chemical. Cells were treated for a total of 48 hours.

RNA isolation and Analysis of Gene Expression Using RT-PCR – For RNA isolation from LS180 cells, 500 μ L of acidic phenol-guanidinium isothiocyanate solution (TRIzol, Invitrogen) was added to each well and total RNA was extracted according to the manufacturer's protocol. cDNA was synthesized from 1 μ g of total RNA (isolated as described) using the iScript cDNA synthesis kit (BioRad) method. For each 20 μ L PCR reaction, 1 μ L of the reverse transcription reaction was used as template, along with 300 nM of UGT1A-specific primers (Strassburg et al., 1998; Strassburg et al., 1999), 5 mM dNTP mix, and 1 unit of Taq (Denville) in proprietary buffer. PCR was performed as described (Chen et al., 2005). 10 μ L of each reaction was visualized on a 1.8% agarose gel containing 1 μ g/mL ethidium bromide. Quantitative real-time PCR was performed on a Stratagene MX4000 Multiplex QPCR. 1 μ L of iScript cDNA product was used in 20 μ L PCR reactions with Mesa Green qPCR MasterMix (Eurogentec, San Diego CA), and each sample was run in triplicate. Quantitative PCR analysis for UGT1A1, UGT1A3, UGT1A4, and UGT1A6 was run with the sense and antisense primers listed in Table 2-1 and described by Senekeo-Effenberger et al, 2007. To determine the quantitation of gene expression, the comparative threshold cycle method (ΔC_T) was used (Livak and Schmittgen, 2001). Mouse carboxypeptidase-H (mCPH), a housekeeping gene, was

used to control for variations in mRNA quantity and quality; subtraction of the mCPH C_t values from the C_t of the *UGT* gene of interest yielded the ΔC_t value. Fold induction was calculated by normalizing ΔC_t values to the average DMSO ΔC_t value.

Mapping the UGT1A4 Transcriptional Start Site – The UGT1A4 transcription start site was mapped using the First Choice RNA ligase-mediated rapid amplification of cDNA ends (RACE) (Ambion) to selectively amplify a full-length capped mRNA of choice. Total RNA was isolated from LS180 cells that had been treated with Rifampicin to increase levels of UGT1A4 transcript. 5 μ g of total RNA was treated with calf intestinal phosphatase, phenol-chloroform extracted, and decapped using tobacco acid pyrophosphatase, according to the manufacturer's protocol. The decapped full-length mRNA was ligated with the provided 5'-RACE adapter oligonucleotide. Reverse transcription followed by PCR using a provided 5' primer specific for the oligonucleotide cap and a 3' primer specific for the UGT1A4 mRNA sequence (5'-tgaattggctcgtagtaact-3') amplified UGT1A4 capped sequences specifically; PCR using this product as a template, an adapter-specific forward primer, and a 1A4-specific reverse primer (5'-cacacaacacctatgaagg-3') resulted in sufficient product to be cloned into a pcR 2.1-TOPO vector (Invitrogen). Clones were screened by restriction digest with XbaI and NcoI, sites for which are specific to UGT1A4 and are not found in UGT1A3 or 1A5. Positive clones were sequenced and the sequence was compared with the oligonucleotide adapter. The nucleotide sequence beginning immediately 3' of the adapter was determined to be the transcriptional start site.

UGT1A4 Promoter Cloning and Expression – Human liver DNA was used as a template for high-fidelity PCR to isolate the 4.964kb *UGT1A4* promoter and -3230, -2969, and -937 bp promoter fragments. Oligonucleotide primers were designed to incorporate restriction sites onto the 5' (NheI) or 3' (XhoI) ends to facilitate cloning into pGL3-basic promoter vectors. Constructs shorter than 900 bp were isolated by PCR using the -937 construct as a template. Primers are listed in Table 3-1. PCR products were first cloned into the pcR-2.1 TOPO vector (Invitrogen, La Jolla, CA) according to the manufacturer's protocol. The *UGT1A4* promoter fragments were excised from the pcR-2.1 TOPO vector by NheI and XhoI digest and were subcloned into the pGL3-basic reporter vector. For analysis of the functionality of the 1A4 promoter DR1 element, complementary oligonucleotides encoding three copies of the *UGT1A4* (5'-ctagGGGGACTAGGGCAATGGTGActag-3') DR1 sequence were synthesized, annealed, and cloned into the SV40pGL3 expression (pGL3-promoter) vector. A 3x concatenated *HMG-CoA Reductase* DR1 element (5'-tcgaTCACCATTGCCCTAGTCCCCctag-3') was also created as a positive control. Mutation of response elements was performed using Quick-Change mutagenesis (Stratagene) according to the manufacturer's protocol (primers are listed in Table 3-1). Mutagenesis was conducted using complementary oligonucleotides containing the 2-bp mutation. PCR was performed for 14 cycles at an annealing temperature of 55⁰C. Presence of the mutations was confirmed by forward and reverse sequencing. All transfection experiments were conducted in triplicate using LS180 cells. Cells were cultured to ~80% confluence in 12-well plates and transfections were performed

Table 3. Oligonucleotides used in cloning the UGT1A4 promoter. Constructs P1-10 were cloned using the forward primers listed and a common reverse primer located at +33 bp past the transcriptional start site. Primers used in Quick-change mutagenesis are listed; reverse complementary primer pairs were used. Introduced mutations are in **bold**.

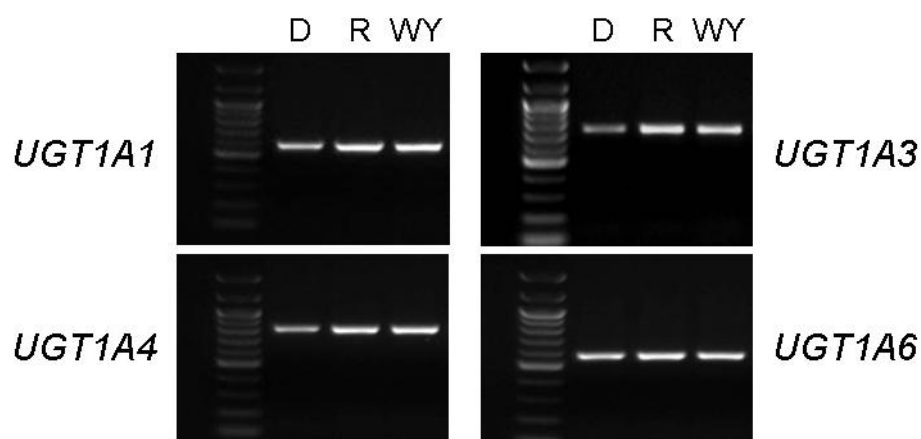
<i>Construct</i>	<i>Oligonucleotide sequence (5' → 3')</i>	<i>Location</i>
P1	ggctctgtattggtgccttta	-4960
P2	catgctgagactccatctct	-3230
P3	gaaaagctgggtgcagtagc	-2969
P4	cctggccataatcttcagga	-937
P5	ccttgctaggggtgtcttgc	-753
P6	aaccagatagccagcctgaa	-540
P7	gcgtcacattcagcagagaa	-418
P8	gaactggatgttcccaga	-257
P9	ttcaagataggcgtgattgg	-212
P10	taatgggtaataagtaact	-150
Reverse	aggggaacctggagtctc	+33
P2m	gcagtgtgccatgttc gt gccacctcactcc	-3106
P4m	tggttgggggactaatgcaatggtgactctc	-387
P4Pm1	ggcagcacagggg g aaaatgagcttttc	-237
P4Pm2	cacagggtcaag gc gagctttcaagata	-232

using 200 ng of plasmid DNA that had been isolated within 3 days prior to transfection along with 5 ng of phRL-SV40 using LipofectAMINE 2000 reagent according to the manufacturer's protocol (Invitrogen). After 24 h, the cells were treated for 48 h with 100 μ M WY-14643, 10 nM TCDD, or 10 μ M Rifampicin dissolved in DMSO or vehicle alone. Dual luciferase assays were conducted according to the manufacturer's instructions (Promega). While the cells were still attached, 200 μ l of Passive Lysis Buffer (Promega) was added, and 20 μ l of lysate was used for dual luciferase analysis using an LMax II³⁸⁴ luminometer. Firefly luciferase values were normalized to *Renilla reniformis* luciferase and protein concentration and reported as -fold increase over vehicle-treated cells.

Results

Several human cell lines were examined for *UGT1* basal and regulatable expression patterns. LS180, a colorectal adenocarcinoma cell line, robustly expressed UGT1A3 and 1A4. Treatment with the human PXR ligand Rifampicin (Rif) or the PPAR α ligand WY resulted in significant induction of UGT1A1, 1A3, 1A4, and 1A6, indicating the presence of an intact signaling pathways in these cells (figure 3-1). Notably, 1A3 and 1A4 were the most strongly induced of the four genes, whereas expression and induction of these isoforms is almost completely absent in HepG2 cells. Quantification of induction by Real-time Quantitative RT-PCR showed a 6.85-fold (1A3) or 5.1-fold (1A4) increase over DMSO when cells were treated with 100 μ M WY, compared to a 2.8-fold increase in UGT1A1. With Rif treatment, 1A3 showed a 10.1-fold increase and 1A4 was induced 6.3-fold, compared to a 5-fold

A.



B.

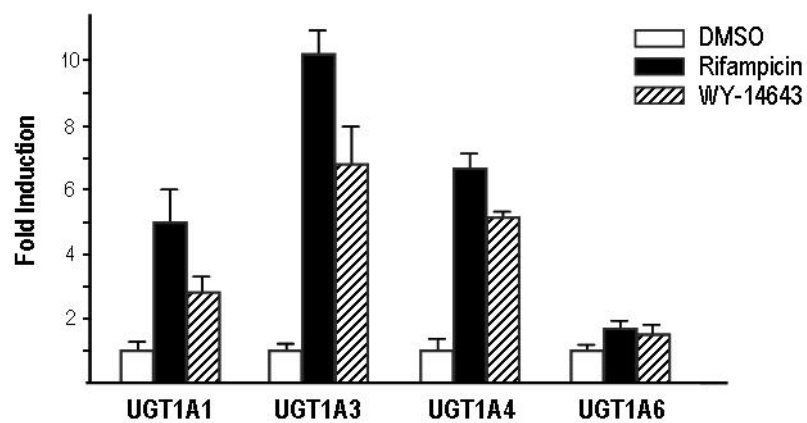


Figure 3-1. Characterization of LS180 Cells as a PXR- and PPAR α -Responsive Cell Line. LS180 cells were treated with either DMSO (D), 10 μ M Rifampicin (R), or 100 μ M WY-14643 (WY) for 48 hours in triplicate. RNA from each well was isolated. A) RNA for each treatment was pooled and RT-PCR was run with isoform-specific primers for 30 cycles. B) Quantitative RT-PCR was run for individual samples. Each sample was normalized to β -actin to control for RNA quality and concentration. Fold Induction is expressed as fold change over DMSO for each isoform.

increase in UGT1A1. LS180 cells were determined to be the cell line of choice for studying UGT1A4 or the closely related UGT1A3. Because LS180 cells express a complement of drug metabolism genes, including *UGT1* in addition to phase I and phase III metabolism genes, this line is routinely used in drug metabolism studies (Gupta et al., 2008).

On the *UGT1* gene locus, approximately 5 kb of regulatory sequence exists upstream of the *UGT1A4* gene. To determine the gene's transcriptional start site, RACE (rapid amplification of cDNA ends) mapping was used, in which nascent mRNA was chemically de-capped and an end-labeled oligonucleotide was hybridized to the RNA in its place. Reverse transcription followed by PCR and then sequence analysis allowed for the identification of the transcriptional start site of the *UGT1A4* gene, located approximately halfway between the TATA motif and the ATG open reading frame (figure 3-2). Upon identification of the transcriptional start site, oligonucleotide primers were designed for amplification of the entire 4.96 kb of *UGT1A4* 5' regulatory sequence, with the 5' primer beginning immediately 3' of the *UGT1A5* exon 1 sequence and the 3' primer extending past the transcriptional start site. A 5 kb product was obtained and cloned into the pGL3 basic luciferase reporter vector for transfection studies.

Putative response elements were identified for the AhR, PXR, and PPAR, as shown in Figure 3-2. A putative PPRE at -4790 was identified within the 5'-most region of the promoter; deletion of this region in construct P2 did not affect induction by WY, indicating that this is not the primary PPRE on the regulatory sequence. Two putative PXRE at -2672 and -2159 do not appear to contribute to overall induction by

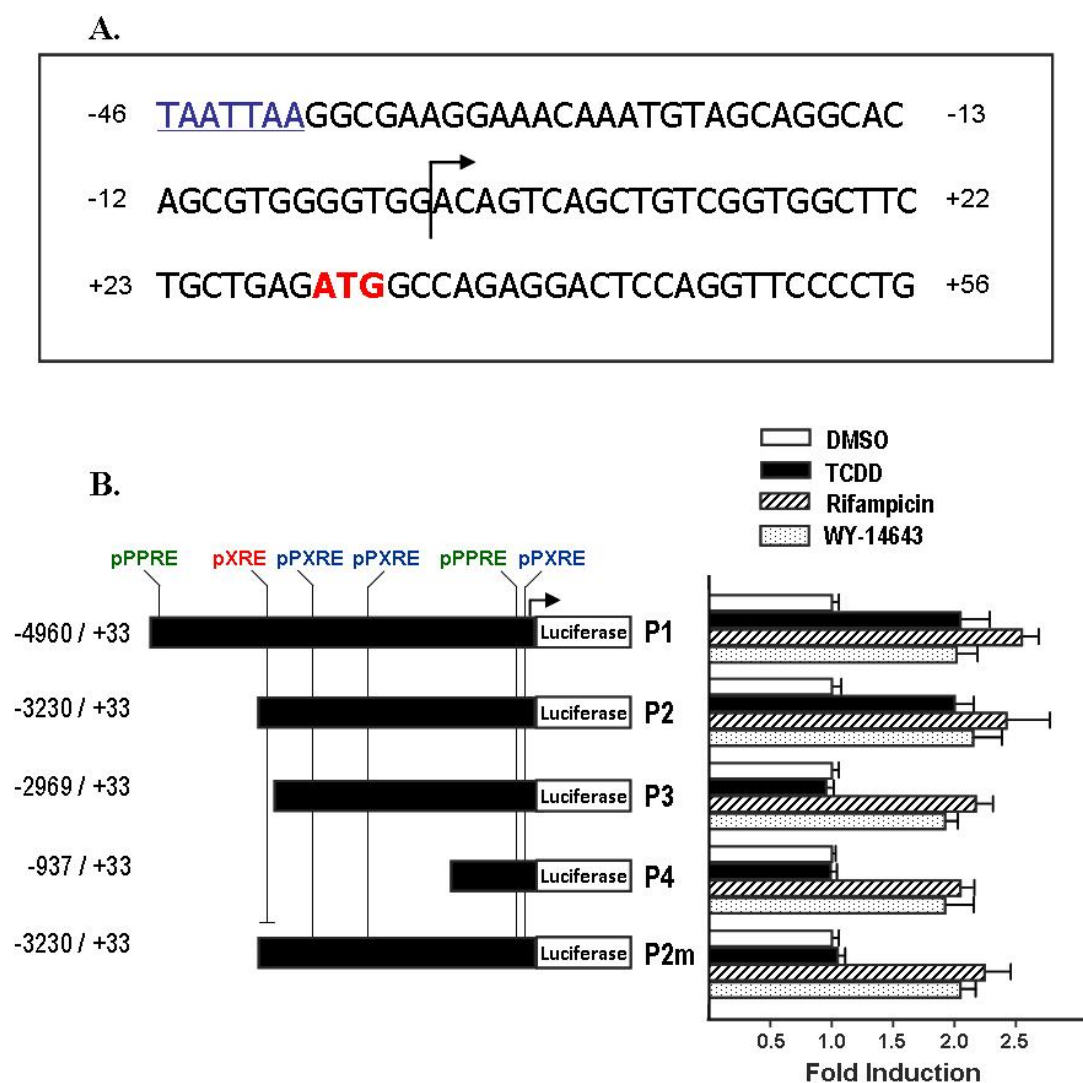


Figure 3-2. Characterization of the *UGT1A4* promoter. **A)** Schematic of the transcriptional elements of the promoter. The *UGT1A4* transcriptional start site was identified approximately halfway between the TATA element (in blue, underlined) and the open reading frame (in red, **bold**). **B)** Putative PPAR response elements (pPPRE) -4790 and -366, putative PXR-response elements at -2672, 2159, and -232, and a putative AhR element (XRE) at -3090 were identified by sequence on the 4.96kb promoter. Promoter fragments isolated by high-fidelity PCR were cloned into pGL3-luciferase reporter vectors and transfected into LS180 cells. 24 hours after transfection, cells were treated with DMSO, TCDD, Rifampicin, or WY-14643 for 48 hours. Luciferase activity was normalized to protein levels. Fold induction for each transfection was calculated by dividing normalized luciferase activity for each treatment by the DMSO activity.

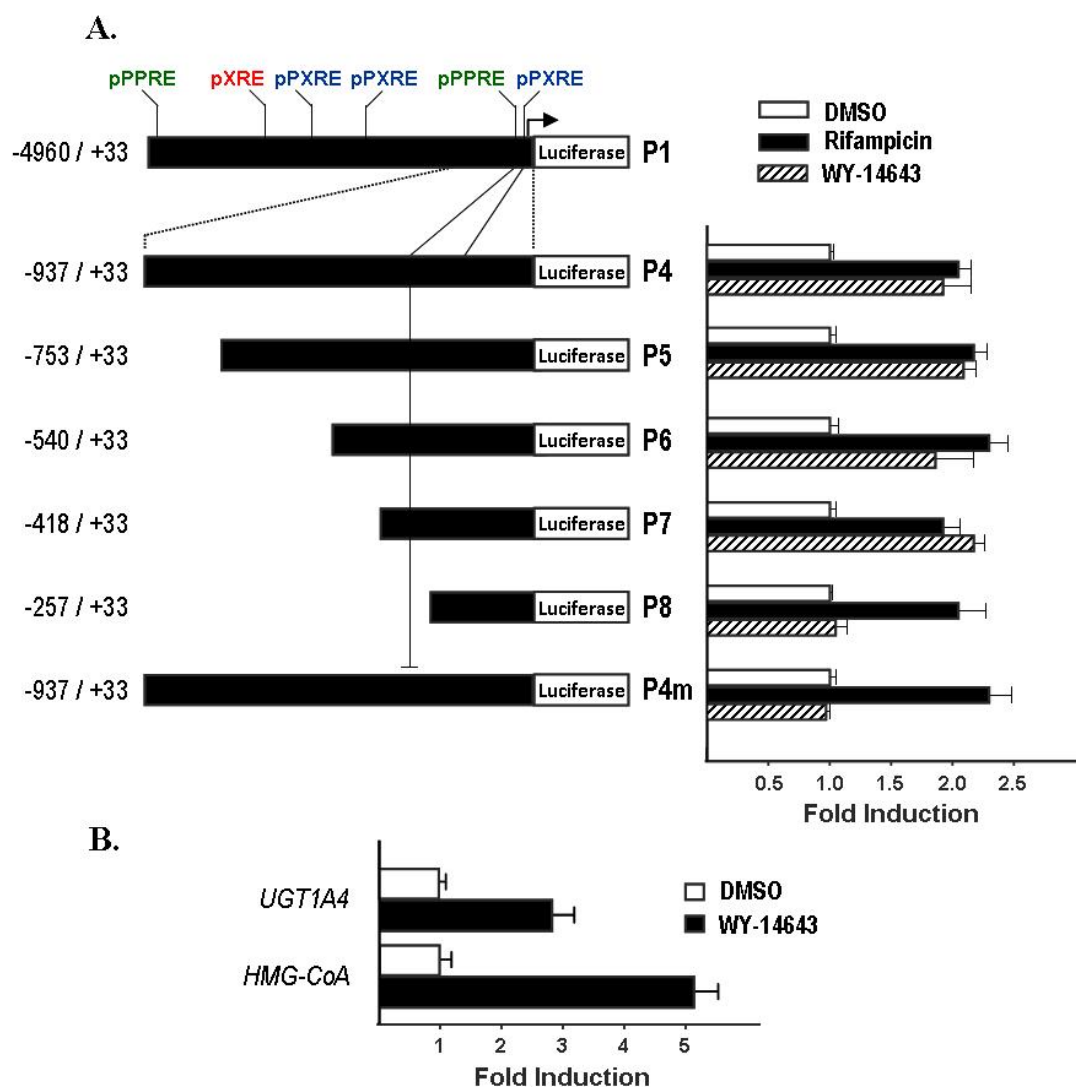


Figure 3-3. Identification of a PPAR α Response Element on the *UGT1A4* promoter. **A)** Promoter fragments isolated by high-fidelity PCR were cloned into pGL3-luciferase reporter vectors and transfected into LS180 cells. 24 hours after transfection, cells were treated with DMSO, 10 μ M Rifampicin, or 100 μ M WY-14643 for 48 hours. Luciferase activity was normalized to protein levels. Fold induction for each transfection was calculated by dividing normalized luciferase activity for each treatment by the normalized DMSO value. **B)** Complementary oligonucleotides containing 3 repeats of the *UGT1A4* DR1 sequence (at -366) or the rat *HMG-CoA Reductase* DR1 were annealed and cloned into SV40-pGL3 reporter plasmids. The constructs were transfected into LS180 cells. The cells were treated with vehicle (DMSO) or WY-14643 (100 μ M) for 48 h followed by analysis of luciferase activity.

PXR: loss of these elements in the P4 construct did not affect induction by the PXR ligand Rifampicin. Induction through the AhR, however, was lost with the P3 construct; TCDD inducibility of the 261bp-longer P2 indicates the existence of an AhR-responsive element in this 261bp region. Mutation of the putative XRE within this segment abolished induction, confirming this sequence as the primary AhR-responsive unit on the *UGT1A4* regulatory sequence.

Serial deletion of the proximal 1 kb, the site of one putative PPRE and one PXRE, revealed a loss of induction by WY on the -257/+33 (P8) promoter construct, which correlates with a deletion past the putPPRE (figure 3-3). Mutation of this DR1 sequence at residues known to be critical for PPAR α binding resulted in loss of induction by WY. To further confirm this DR1 sequence as a PPRE, oligonucleotides consisting of three repeats of the putPPRE were annealed and cloned into the pGL3 promoter vector. The DR1 PPRE from a known PPAR α -responsive gene, rat *HMG-CoA Reductase*, was cloned in a similar way and used as a positive control. Transfection of these constructs and treatment with WY resulted in a modest (2.8-fold) but significant induction with the 1A4PPRE; induction with the positive control was slightly higher (5.1-fold), as might be expected given the robust response to PPAR α demonstrated by rat HMG-CoA Reductase in vivo.

Induction through PXR was maintained on the P9 construct, which encompassed the putative PXRE at -232. Further deletion of the promoter resulted in a relative increase in basal luciferase activity, though no further induction was seen with Rifampicin treatment (figure 3-4). This phenomenon was observed with both the P9 and P10 constructs. When the P4 construct, which demonstrated ~2 fold

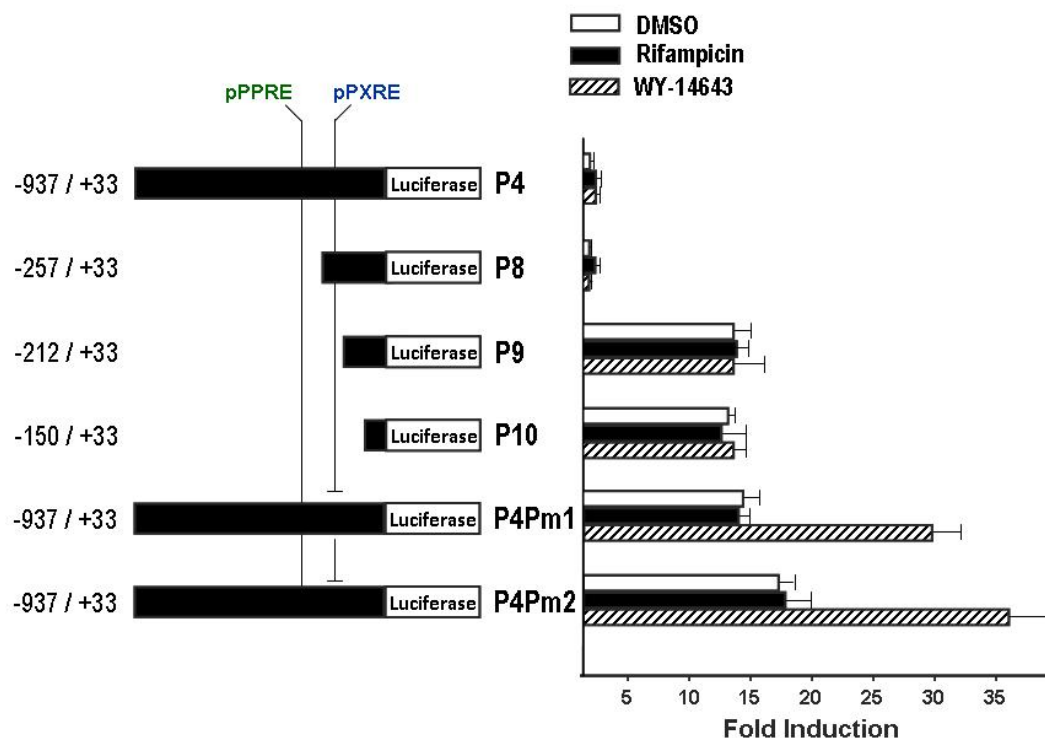


Figure 3-4. Characterization of PXR Induction on the *UGT1A4* promoter. Promoter fragments in pGL3-luciferase reporter vectors were transfected along with a phRL-SV40 Renilla luciferase plasmid into LS180 cells. 24 hours after transfection, cells were treated with DMSO, 10 μ M Rifampicin, or 100 μ M WY-14643 for 48 hours. Firefly luciferase activity was normalized to Renilla luciferase activity and protein levels. Normalized DMSO values for the P4 construct were used to calculate fold induction for each transfection and treatment.

induction with Rif and WY, was subject to mutation of the PXRE (constructs P4Pm1 and P4Pm2), normalized basal luciferase activity increased 13- to 17-fold over the non-mutated parent construct. Induction by WY was 2-2.5 fold over DMSO for these individual transfections, but approximately 30 fold over the DMSO values for the non-mutated parent. No further increase was observed with Rif treatment in these

transfections, indicating that the PXRE mutations were sufficient to abolish PXR induction.

Discussion

UGT1A4 plays a unique and important role in the metabolism of many clinically used drugs, and much attention has been paid to possible regulatory pathways that govern its expression and induction. It has been observed in Tg-*UGT1* mice that PXR, AhR, and PPAR α are all capable of inducing UGT1A4 expression (Chen et al., 2005; Senekeo-Effenberger et al., 2007); however, until this point, studies to determine the site of action of these pathways on the gene's regulatory region have been impeded by a lack of appropriate cell culture model system. Through RT-PCR, *UGT1* gene expression profiles were examined in cell lines commonly used for drug metabolism studies. LS180, a colorectal adenocarcinoma cell line that possesses an intact PXR signaling pathway (Gupta et al., 2008), expressed both UGT1A3 and UGT1A4 at basal levels similar to those observed for UGT1A1 and 1A6 (figure 3-1). UGT1A3 and 1A4 were also robustly induced by treatment with the human PXR agonist Rifampicin. Thus, this line was chosen for further studies on *UGT1A4* regulation.

Identification of the UGT1A4 transcriptional start site allowed for isolation of the complete *1A4* regulatory sequence, including all proximal promoter elements. The 5' end of the regulatory region was determined to begin immediately 3' of the *UGT1A5* exon 1 element, and a 4,964 bp segment extending 3'-ward from this point to just past the transcriptional start site was isolated by high-fidelity PCR and was

cloned into a pGL3 basic luciferase reporter vector. Transfection of this construct into LS180 cells and treatment with PXR, AhR, and PPAR α agonists revealed that responsive elements for all three nuclear receptors were contained in this approximately 5 kb regulatory region (figure 3-2). Through serial deletions, it was determined that the AhR responsive element (XRE) was located within the -3.0 to -3.23 kb region. Sequence analysis of this region identified a candidate XRE whose core sequence, 5' - CACGCC - 3', is a near perfect match for the UGT1A1 XRE (5' - CACGCA - 3') (Yueh et al., 2003). Mutation of the core sequence resulted in a complete loss of induction by the AhR ligand TCDD, confirming this sequence as the primary XRE in the *UGT1A4* regulatory region. In contrast to these findings, two XRE in the *1A4* proximal promoter region, at -163 and -219, were recently identified as the primary AhR-responsive elements (Erichsen et al., 2008). This discrepancy can be explained, in part, by the difference in overall length of intact regulatory sequence analyzed; in the referenced study, only the proximal 300 bp were analyzed for response. This presents an interesting question of whether, by deleting regulatory elements 5' of this sequence, overall response and inducibility of the construct could be affected. In this study, it is demonstrated that -3.0 kb and -937 bp constructs are not responsive to TCDD. Could a suppressive element in the sequence between -937 and -300 explain the differing results?

Elements within the proximal 500 bp of the *UGT1A4* promoter are also responsible for induction through PXR and PPAR. Putative PXRE and PPRE were identified by sequence at -4790 (PPRE), -2672 (PXRE), -2159 (PXRE), -366 (PPRE), and -232 (PXRE). Although a slight loss of induction by PXR and PPAR α was

observed with the P3 and P4 constructs, significant increases in luciferase activity upon Rif or WY treatment were still recorded (figure 3-2). Further deletion into the proximal 900 bp produced -753 (P5), -540 (P6), -418 (P7), -257 (P8), -212 (P9), and -150 (P10) promoter constructs (figures 3-3, 3-4). WY induction was lost with P8, indicating the presence of the primary PPRE in the -257 to -418 promoter segment. The sequence identified as a putative PPRE at -366 correlates with these findings. Mutation of this -366 DR1 element resulted in loss of WY induction. Furthermore, a concatenated -366 response element was induced by WY treatment in LS180 cells, confirming this DR1 element as a PPRE (figure 3-3).

Induction of these promoter reporters by Rif was maintained through the P8 construct. Loss of Rif induction on the P9 and P10 constructs was observed, which correlates with the loss of the putative PXRE at -232. Curiously, the basal luciferase activity increased greatly with the P9 and P10 constructs in comparison to plasmids containing longer promoter elements. Mutation of the PXRE at -232 abolished induction by Rif, but also caused an increase in the basal luciferase activity in comparison to the non-mutant parent. Generation of a second PXRE mutant produced similar results. These results suggest that unliganded PXR may act through the -232 PXRE to suppress transcription of 1A4, a heretofore uncharacterized role for the Pregnane X Receptor in control of the *UGT* genes and drug metabolism in general. The corepressor SHP interacts with many of the nuclear receptors, including ER (Seol et al., 1998), AR (Gobinet et al., 2001), AhR/Arnt (Klinge et al., 2001), LXR (Brendel et al., 2002), FXR (Goodwin et al., 2000), and RXR (Lee et al., 2000), inhibiting transactivation by blocking the interaction of nuclear receptors with DNA

response elements. Unlike many of the other adopted orphan nuclear receptors, however, PXR has been shown to interact simultaneously with DNA response elements and corepressors in the absence of ligand (Synold et al., 2001; Hariparsad et al., 2009; Ourlin et al., 2003), suggesting a possible role for PXR as a basal and unliganded suppressor of gene transcription. There is also evidence that PXR may actively suppress target genes through interaction with an inhibitory ligand (Synold et al., 2001; Tabb et al., 2004; Huang et al., 2007), though thus far an endogenous PXR inhibitory ligand has not been identified.

These data represent the first molecular evidence that PXR may act on UGT1A4 in a suppressive manner, and identification of a suppressive element on the *UGT1A4* promoter may reconcile conflicting data regarding nuclear receptor activation of the *UGT1A4* gene.

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CHAPTER 4

Hormonal Regulation of the Human *UGT1* Locus during Estrus

Introduction

The endocrine signaling that accompanies growth and reproduction is remarkable in its scope and it affects almost every process in the body. Drug metabolism profiles change dramatically during these global hormonal changes, and glucuronidation is not an exception. The developmental patterns of human UGT expression are well characterized, with UGT activity, nearly undetectable in the fetus and very low in neonates, surging to near-adult levels within 20-30 weeks after birth [reviewed in (DUTTON, 1978)](Burchell et al., 1989).

The regulation of the UGTs during reproduction, however, is less clear. There is compelling clinical evidence suggesting that UGT1As, and UGT1A4 in particular, are induced during pregnancy. Lamotrigine, an antiepileptic drug cleared primarily through glucuronidation by 1A4, exhibits increased clearance during pregnancy (Tran TA et al., 2002;Pennell et al., 2004), leading to increased risk of seizure (Pennell et al., 2008). Several studies determined clearance to increase 200-300% over nonpregnant values (Pennell et al., 2004;de Haan et al., 2004), and mothers on lamotrigine or similar drug therapy must be monitored throughout pregnancy to ensure maintenance of proper plasma concentrations of this critical drug. There is also clinical evidence that increases in hormone levels due to oral contraceptive steroids (OCS) are sufficient to alter drug metabolism profiles. In one study, lamotrigine clearance was found to increase 2-3 fold in patients taking oral contraceptives, which is remarkably similar to the increase observed during pregnancy (Sabers A et al., 2003). Paracetamol (acetaminophen) clearance increased

50% in women receiving OCS, resulting from an increase in the glucuronide formation (Miners JO et al., 1983), and clearance and glucuronide formation of the hypolipidemic drug clofibrate were also increased with OCS intake (Miners JO et al., 1984;Liu HF et al., 1991).

However, as it is not possible to perform regulatory studies on pregnant women due to ethical considerations, most of the information regarding drug metabolism during pregnancy has been obtained using laboratory animals and the rat in particular. Examination of phase I enzymes during pregnancy showed a downregulation of many *Cyp* genes, with the notable exception of *Cyp3a1* (He et al., 2005;Dickmann et al., 2008;He XJ et al., 2006;Masuyama H et al., 2001), which metabolizes estradiol (Suchar et al., 1996). Interestingly, analysis of pregnant rat livers showed a decrease in *Ugt1a* protein and activity compared to nonpregnant or postpartum females (Luquita MG et al., 2001;Matsumoto J et al., 2002), which contradicts the human clinical data. When the livers of pregnant Tg-*UGT1* mice were analyzed, dramatic increases were observed in UGT1A1, 1A4, and 1A6 protein (Chen et al., 2005), indicating that UGT induction during pregnancy is an event specific to the human *UGT1* genes, although the signal transduction pathways that mediate the induction are clearly intact in the mouse.

In the mouse, serum progesterone and estrogen reach peak levels of 100 ng/mL (~500 nM) and 85 pg/mL (500 pM) in the last week of pregnancy (Masuyama H et al., 2001). Although mouse PXR is not as effectively activated by estrogens as the human receptor, progesterones have been shown to activate mPXR at concentrations in the 10-100 nM range, 5-10 fold lower concentrations than those

reached during pregnancy. PXR is also expressed in many reproductive tissues as well as the liver and intestines (Kliwer SA et al., 1998) and is highly upregulated in the livers of pregnant mice in a manner that correlates temporally with induction of the PXR target gene *Cyp3a* (Masuyama H et al., 2001) and the increases in UGT1A protein observed in the Tg-*UGT1* mice. These data suggest that PXR may act as a sensor of pregnancy hormones and mediate the observed UGT1A induction.

Although the hormonal fluctuations during the mouse estrus or the human menstrual cycles are not as large as those seen during pregnancy, these fluctuations occur far more frequently, and thus any changes in drug metabolism profiles during these cycles could be of clinical relevance. Aside from reports that rat *Cyp1b1*, expressed largely in steroidogenic tissues, is highly upregulated in the rat ovary during proestrus, little is known about the effect of estrus on drug metabolism. Plasma levels of progesterone and estradiol fluxuate throughout the rodent's 4-day cycle, with circulating progesterone concentrations reaching 15 ng/mL (~75 nM).

To examine the impact of the estrus cycle on *UGT1* transcript levels, the estrus cycles of age-matched Tg-*UGT1* mice were observed, and liver samples were collected from animals at each stage. The contribution of PXR to this regulatory pattern was examined by breeding the Tg-*UGT1* mice into a PXRnull background (Xie et al., 2000a) to generate a Tg-*UGT1*-PXRnull line.

Materials and Methods

Generation of mouse lines – mPXR^{+/-} mice were obtained from Dr. Ronald M. Evans [The Salk Institute, La Jolla CA; (Xie et al., 2000a)] and were bred to a homozygous

mPXR^{-/-} genotype (PXRnull) in a C57BL/6N background. Genotype was determined using primers directed to the target recombination site. Wild type was identified with an upstream primer (5' AGAAACACATAGAAACCCATCCATG) in combination with a downstream primer (5' AGTCCACCAAGCCTGAGCCTCCTAC) yields a 472 bp product in wt mice. The same upstream primer in combination with a Neo-specific primer (5' CTTGACGAGTTCTTCTGAGGGGATC) produces a 521 bp product that identifies the PXRnull genotype. Tg-*UGT1* mice were generated and genotyped as described (Chen et al, 2005) and were bred six generations into a C57BL/6N background. The Tg-*UGT1* and PXRnull lines were crossed to achieve a *UGT1*^{+/-}*PXR*^{+/-} genotype; these mice were then interbred to obtain a *UGT1*^{+/-}*PXR*^{-/-} line.

Determination of Estrus – 3-month-old female Tg-*UGT1* and Tg-*UGT1*-PXRnull mice were housed in a light- and temperature-controlled facility, one mouse per cage. To synchronize cycling, a small sample of dirtied litter from cages housing male mice was placed into each female's cage. Stage of estrus was monitored daily within the first two hours of the light cycle to minimize any possible circadian effects. Mice were douched with a small amount of warm water using a blunted Pasteur pipette in order to collect cells from the vagina. Douche samples from each mouse were analyzed by light microscopy and estrus stage was determined by cell morphology (see Figure 4-1). Only mice that showed at least three regular 4- or 5- day estrus cycles were used. Mice were sacrificed within an hour of douching; 3 mice were sacrificed per stage (Diestrus I, Diestrus II, Proestrus, and Estrus). Livers were

isolated, pulverized under liquid Nitrogen for homogeneity, and stored at -80°C . Total RNA was isolated using the TRIzol method (Invitrogen) and approximately 100 mg of pulverized tissue.

Analysis of RNA – cDNA was synthesized from 1 μg of total RNA using the iScript cDNA synthesis kit (BioRad) method. PCR was performed using isoform-specific primers (listed in Table 2-1) for 30 cycles. Quantitative real-time PCR was performed on a Stratagene MX4000 Multiplex QPCR. 1 μL of iScript cDNA product was used in 20 μL PCR reactions with Mesa Green qPCR MasterMix (Eurogentec, San Diego CA), and each sample was run in triplicate. Quantitative PCR analysis for UGT1A1, UGT1A3, UGT1A4, UGT1A6, and UGT1A9 was run with the sense and antisense primers listed in Table 2-1 and described by Senekeo-Effenberger et al, 2007. Mouse carboxypeptidase-H (mCPH), a housekeeping gene, was used to control for variations in mRNA quantity and quality. Fold induction was calculated by normalizing each sample to untreated adult male liver RNA.

Results

Mouse estrus is a 4- to 5-day cycle and involves a 1-day Diestrus I (Metestrus), a 1-2 day Diestrus II (Diestrus), a 1-day Proestrus, and a 1-day Estrus. For each of the cycling female mice, the stage of estrus was determined by examination of cell morphology from douchings performed daily (figure 4-1). Diestrus I is characterized by a predominance of small leukocytes with a few

remaining collapsed cornified cells (squames) in the vaginal douche, with decreasing levels of estradiol and progesterone. Diestrus II, also referred to as the Luteal Phase,

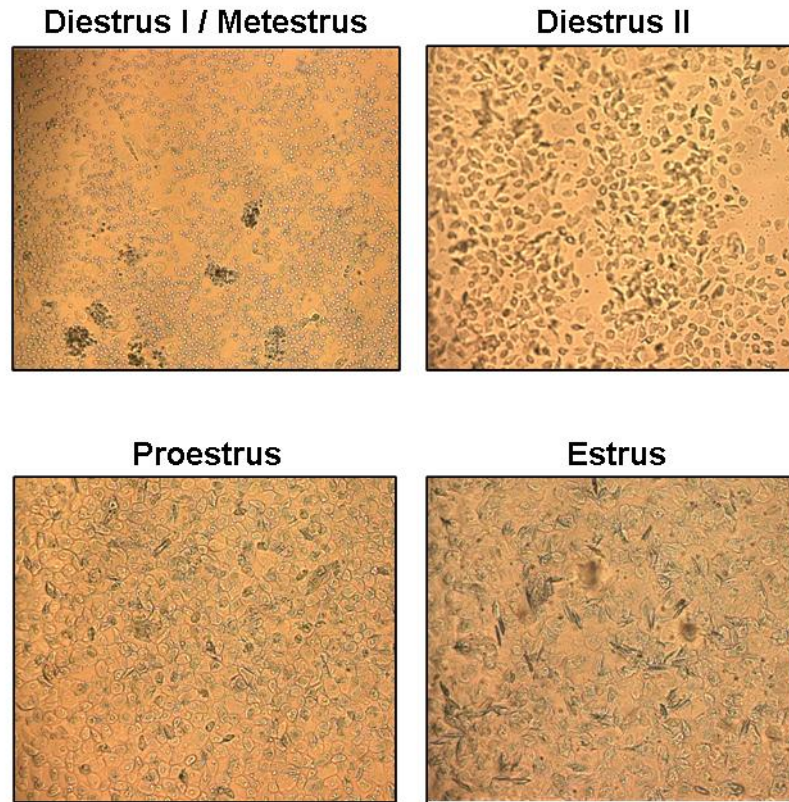


Figure 4-1. Cell Morphology of the Estrus Cycle. Warm water douches from female cycling Tg-*UGT1* and Tg-*UGT1*-PXRnull mice were examined under light microscopy for cell type and morphology. Estrus stage was assigned accordingly. Images shown represent a 10x magnification, $\frac{1}{4}$ field-of-view crop.

is dominated hormonally by progesterone and is characterized by stringy mucous containing some leukocytes and many small nucleated epithelial cells.

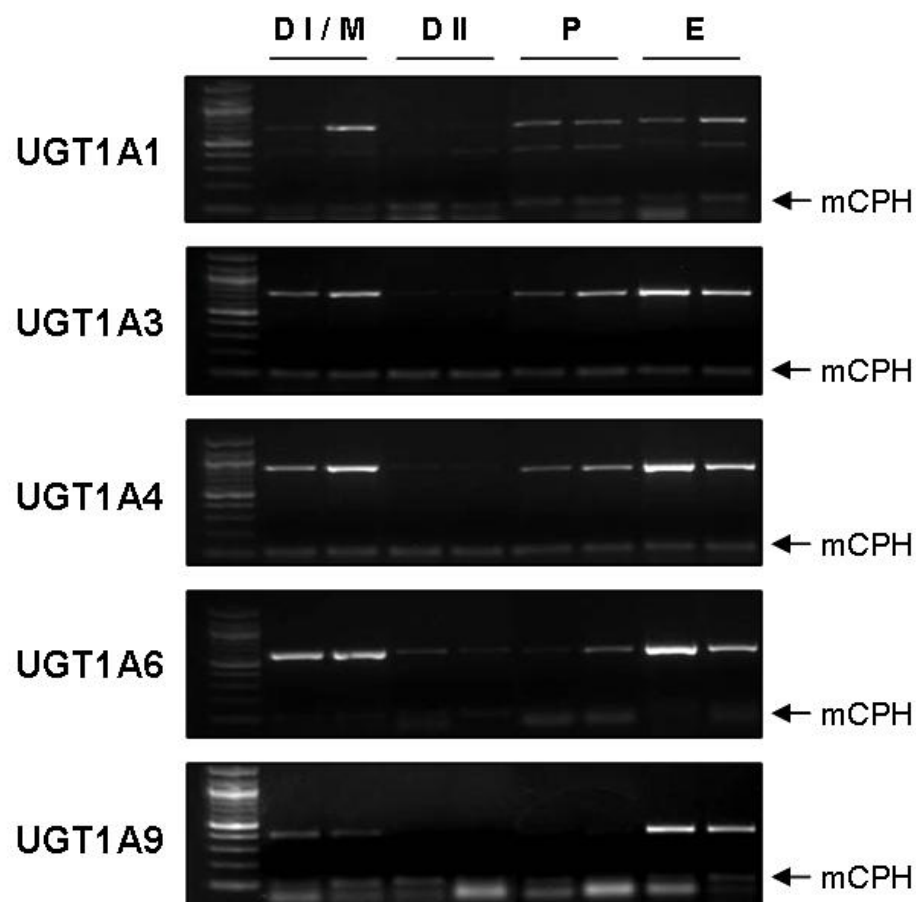


Figure 4-2. The *UGT1* Genes are Regulated During Estrus. RNA was isolated from the livers of cycling female Tg-*UGT1* mice at varying stages of estrus: Diestrus I / Metestrus (D I / M), Diestrus II (D II), Proestrus (P), or Estrus (E). Three or more animals were sacrificed per group; two representative samples are shown for each stage. Reverse transcription followed by 30 cycles of PCR using isoform-specific primers was performed, using mCPH as an internal control.

Proestrus represents the peak of estrogen and a falling off of progesterone; the growth of the follicle during this stage is why it is also known as the Follicular Phase. Vaginal cell morphology during proestrus consists of sheets of round-to-irregular nucleated cells and no leukocytes. Ovulation occurs approximately 10 hours after the onset of Estrus, during which time progesterone levels are low and estrogen is falling. Pre-ovulation, the vaginal cells are large and irregular with degenerate nuclei; as Estrus progresses, the cornified cells collapse and clump together. Most mice exhibit regular 4- or 5-day estrus cycles with cell morphologies as described. Mice that did not display at least 3 regular cycles in a row were not used in this study.

Regulation of UGT1A1, 1A3, 1A4, and 1A6 was dynamic during estrus, with downregulation of all transcripts observed during Diestrus II (figure 4-2). During Proestrus, transcript levels had increased relative to the Diestrus II levels, and peak transcript levels occurred during Diestrus I / Metestrus or Estrus for all isoforms analyzed. Quantification of transcriptional levels through real-time RT-PCR confirmed this pattern of regulation (figure 4-3). When the transcript levels of the Diestrus II samples were normalized to adult male liver, UGT1A1, 1A3, 1A4, and 1A9 were 16.7, 6.25, 4.35, and 1.3 fold lower, respectively, than the male liver, representing a dramatic downregulation during this phase. Transcript levels recovered during Proestrus, with UGT1A1 remaining 1.4-fold lower than the male, while UGT1A3, 1A4, 1A6, and 1A9 were 2.7, 2.2, 1.8, or 2.6-fold higher, respectively. Estrus, however, represented a peak in UGT induction during the cycle, with 5.4-fold (UGT1A1), 15.3-fold (UGT1A3), 16.9-fold (UGT1A4), 5.9-fold

(UGT1A6), and 16.6-fold (UGT1A9) increased expression compared to the male mouse liver.

To investigate the contribution of PXR to the regulation of the *UGT1* genes during estrus, Tg-*UGT1*-PXRnull mice were monitored for estrus cycling and sacrificed at each stage (figure 4-3).

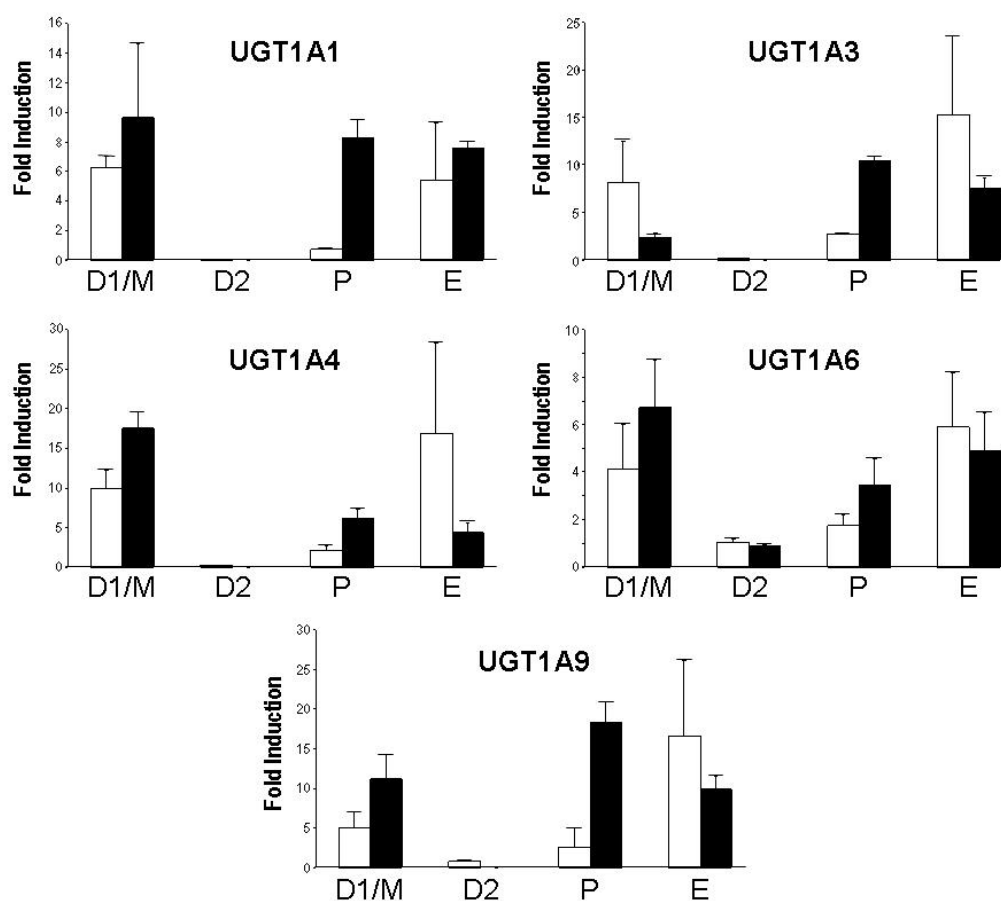


Figure 4-3. The Role of PXR in UGT1 Regulation During Estrus. RNA was isolated from the livers of cycling female Tg-*UGT1* (white bars) and Tg-*UGT1*-PXRnull mice (black bars) at varying stages of estrus: Diestrus I / Metestrus (D1/M), Diestrus II (D2), Proestrus (P), or Estrus (E). Three or more animals were sacrificed at each stage. Quantitative RT-PCR was performed using isoform-specific primers and mCPH primers to control for RNA quality and concentration. Each sample was performed in triplicate and was normalized to values obtained from adult male liver.

The Tg-*UGT1*-PXRnull mice showed a similar pattern of regulation as Tg-*UGT1*, with a downregulation of transcription during the Diestrus II phase. Interestingly, the fold downregulation in the PXRnull background was even greater than that observed in the Tg-*UGT1* line, with 100-fold lower UGT1A1, 140-fold lower UGT1A3 and 1A4, and 33-fold lower UGT1A9 than in male liver. Also in contrast with the pattern observed in Tg-*UGT1* liver was a large upregulation during Proestrus, especially in UGT1A1, 1A3, and 1A9. The overall fold of induction did not differ significantly during Diestrus I or Estrus from the Tg-*UGT1* samples.

Discussion

There is clear evidence that the global hormonal changes that occur during development, pregnancy, and lactation are sufficient to induce dramatic alterations in glucuronidation. To explore whether the hormonal signaling experienced by Tg-*UGT1* mice during the estrus cycle could also regulate *UGT1* gene expression, experiments were undertaken to examine UGT1 transcript levels during the various stages of estrus. By monitoring the morphology of cells obtained by daily vaginal douches, it was possible to ascertain a mouse's progress through estrus (figure 4-1), and mice were sacrificed accordingly.

Regulation of the human *UGT1* genes throughout the cycle was striking. Mice exhibited a dramatic downregulation of all liver-expressed UGTs during the Diestrus II phase (figure 4-2). When quantified, the levels of UGT1A1, 1A3, and 1A4 were much lower than those observed even in male mouse liver (figure 4-3). As Tg-*UGT1* mice express the human *UGT1* genes in a gender-dimorphic manner, with

significantly lower basal transcript levels of UGT1A1, 1A3, 1A4, and 1A6 in male liver (data not published), this finding was surprising. During Proestrus, expression returned to approximately 1-2 fold above male. Peak levels of transcript were seen during Estrus and Diestrus I, which represented a ~5-fold increase over male levels for UGT1A1 and 1A6. UGT1A3 and 1A4, which have previously been shown to be expressed basally at a lower level than either 1A1 or 1A6 but which also are highly induced by the nuclear receptors in Tg-*UGT1* liver, are also highly induced during the Estrus stage: 1A3 expressed at a 15-fold higher level than in males, and 1A4 was 17-fold higher. Surprisingly, even UGT1A9, which is normally more highly expressed in male mice, was induced 16.6-fold during Estrus. Although these values were normalized to UGT expression levels in adult male mouse liver, it is of note that the fold change between Diestrus I and Diestrus II was even more striking. For UGT1A1, Diestrus I transcript levels were 110-fold higher than in Diestrus II. UGT1A3 demonstrated a 50-fold change, while UGT1A4 expression was 40-fold greater in Diestrus I than just one day later in Diestrus II.

Despite the dramatic regulation observed in these mice, it is difficult to predict how these results might translate to human health. Clinically, any potential changes in levels of drug metabolizing enzymes could have implications for drug-drug interactions. However, there are vast differences in cycle length between mice, which have 4- or 5-day cycles, and humans, which have 25- to 35-day cycles. In mice, the hormonal changes that induce endometrium thickening and ovulation are rapid and transient, and although transcriptional levels in Tg-*UGT1* mice are highly up- or down-regulated, these changes, too, are transient. In these mice, the overall change in

UGT protein concentration or activity may only be slight because of this rapid progression through the cycle and the relatively slow protein turnover. However, circadian expression of mouse or rat *Ugt* transcripts vary several fold throughout the light and dark cycles and this is sufficient to cause temporal changes in xenotoxicity which are clinically quite relevant, particularly when timing doses of drug with low therapeutic indices such as cancer chemotherapeutics (Zhang et al., 2009; Belanger et al., 1985; Labrecque and Belanger, 1991). In humans, the reproductive cycle is greatly extended, involving a 6-16 day follicular phase in which Estradiol levels steadily increase and a 14 day luteal phase that is predominated by Progesterone. The observed transcriptional changes in the Tg-*UGT1* mice may therefore translate into less transient and more clinically significant changes in protein levels and overall glucuronidation activity.

Estradiol and Progesterone are the primary circulating hormones involved in both pregnancy and estrus and are also both ligands for the Pregnane X Receptor, although transactivation of PXR by these hormones is much less efficient than with the Estrogen or Progesterone Receptors. ER has an EC_{50} of 0.5-0.8 nM for the estrogens, estradiol (E_2), and estrone (E_1) and the EC_{50} for transactivation of PR by progesterone is approximately 1 nM. By contrast, PXR has an EC_{50} of 1-10 μ M for progesterone or estrone metabolites, and 20-50 μ M for progesterone, estradiol, and estrone. Thus, PXR is clearly not the most effective mediator of hormone response. However, to this point, there is no direct evidence that ER or PR signaling results in induction of *UGT* genes. PXR, on the other hand, plays an essential role in the regulation of drug metabolizing genes, including the UGTs, and controls induction of

all nine UGT1A isoforms. Moreover, PXR transactivates target genes such as *Cyp3a* at concentrations of progesterones and estrogens that are far below those reached physiologically during pregnancy or with use of oral contraceptives (Masuyama H et al., 2001). It was therefore hypothesized that PXR acts as the primary mediator of hormonal control of the *UGT1* genes.

To investigate this hypothesis, PXRnull mice were bred with Tg-*UGT1* mice to develop a Tg-*UGT1*-PXRnull line. These mice were used in experiments to monitor estrus cycles and liver tissues were collected at each stage. These samples showed a similar pattern of regulation to the livers of Tg-*UGT1* mice, with a large downregulation of transcript levels during Diestrus II. It is apparent from these experiments that PXR does not therefore play a major role in hormonal regulation of the *UGT1* genes, as loss of PXR was hypothesized to result in loss of this regulatory phenomenon. It is interesting, however, to note that Diestrus II levels in these mice were even lower than those observed in Tg-*UGT1*. Conversely, transcript was much higher in the PXRnull background during Proestrus, while there was no significant difference between Estrus and Diestrus I levels in the two mouse strains. These results suggest that PXR may play another kind of regulatory role in *UGT1* gene control, in which PXR controls the extent of up- or down-regulation of the locus, in either a direct or indirect manner.

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CHAPTER 5

PXR is a Master Regulator of the *UGT1* Locus in vivo

Introduction

Among the nuclear receptors, PXR has a key role in the regulation of drug metabolism. PXR is the primary nuclear receptor (NR) governing control of CYP3A4 (Kliewer SA et al., 1998;Lehmann et al., 1998;Blumberg et al., 1998), which metabolizes more than 50% of clinically used drugs (Guengerich, 1999) and also regulates phase II and III drug metabolism (Xie et al., 2003;Vyhlidal et al., 2004;Chen et al., 2005;Synold et al., 2001;Kast et al., 2002). PXR is unique among the NR superfamily because of its ability to respond to a wide range of xenobiotic and endogenous compounds that are diverse in class, size, and structure. Acting as a xenobiotic sensor, PXR activates genes responsible for detoxification and metabolism, but is also involved in homeostatic turnover of bile acids (Staudinger et al., 2001a;Xie and Evans, 2001) and is essential for protection against lithicholic acid-induced liver damage (Staudinger et al., 2001b).

Traditionally considered to exist in an unliganded chaperone-bound monomeric state in the cytosol, upon activation by ligand the NR was thought to shed its chaperone proteins and translocate to the nucleus where it would homodimerize or heterodimerize with RXR α and then activate target genes. A new model has developed in the last few years, however, in which unliganded dimers can exist in the nucleus, interacting with DNA response elements and corepressor proteins (Aranda and Pascual, 2001;Rosenfeld and Glass, 2001) such as SHP and histone deacetylases. Activation by a ligand is thought to cause a dissociation of corepressors and activation of coactivator proteins through NR activation domains such as AF-1 and AF-2 (Glass and Rosenfeld, 2000;Rosenfeld and Glass, 2001;Teotico et al., 2008),

and the combination of diverse genetic sequences with cell type-specific coactivators and corepressors is believed to contribute to the tissue-specific expression patterns of NR target genes.

Structurally, PXR is an atypical nuclear receptor in that it contains a 50-60 amino acid insert that results in a β -turn- β homodimerization motif not found in other nuclear receptors (Teotico et al., 2008; Kliewer SA et al., 1998; Noble et al., 2006) that results in formation of a PXR homodimer and subsequent suggested heterotetramer with RXR (Noble et al., 2006). This heterotetrameric form, in addition to the uniquely high degree of flexibility of PXR's AF2 domain, may underlie a unique function for PXR, allowing it to act as an unliganded repressor by binding both DNA target sequences and corepressors simultaneously (Synold et al., 2001). Evidence presented in this dissertation point toward this role for PXR in control of *UGT1A4* in vitro (Chapter 3) and all liver-expressed UGT1 isozymes in vivo (Chapter 4). In this study, experiments were undertaken to examine the contribution of PXR to basal homeostatic and induced expression of the *UGT1* genes. Using Tg-*UGT1* mice developed in the laboratory (Chen et al., 2005), mice expressing no functional PXR (Xie et al., 2000a), and mice expressing a constitutively active PXR under control of an albumin promoter, new genetic lines were developed to answer questions about the contribution of PXR to expression of the *UGT1* genes and their induction through other nuclear receptors.

Materials and Methods

Reagents – 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was obtained from Wellington Laboratories (Guelph, Ontario, Canada). Pregnenolone-16 α -carbonitrile (PCN) and 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) were obtained from Sigma-Aldrich, Inc.

Generation of mouse lines – mPXR^{+/-} mice were obtained from Dr. Ronald M. Evans [The Salk Institute, La Jolla CA; (Xie et al., 2000a)] and were bred to a homozygous mPXR^{-/-} genotype (PXRnull) in a C57BL/6N background. Genotype was determined using primers directed to the target recombination site. Wild type was identified with an upstream primer (5' AGAAACACATAGAAACCCATCCATG) in combination with a downstream primer (5' AGTCCACCAAGCCTGAGCCTCCTAC) yields a 472 bp product in wt mice. The same upstream primer in combination with a Neo-specific primer (5' CTTGACGAGTTCTTCTGAGGGGATC) produces a 521 bp product that identifies the PXRnull genotype. Tg-*UGT1* mice were generated and genotyped as described (Chen et al, 2005) and were bred six generations into a C57BL/6N background. The Tg-*UGT1* and PXRnull lines were crossed to achieve a *UGT1*^{+/-}-PXR^{+/-} genotype; these mice were interbred to a *UGT1*^{+/-}-PXR^{-/-} line. *Alb*-VPhPXR (*alb*-VPhPXR^{+/-}-mPXR^{+/-}) mice were also obtained from Dr. Evans (Xie et al., 2000a) and were bred with PXRnull mice several generations to produce an *alb*-VPhPXR^{+/-}-mPXR^{-/-} line. These mice contain human *PXR* cDNA fused to an SV40 intron/poly(A) sequence; expression of this construct is under control of the mouse albumin promoter. These mice express moderately high levels of constitutively active hPXR. Genotyping of this line was performed as described for the PXRnull line in

addition to using primers directed to the hPXR cDNA (Forward: 5' GTCCTTGGGGTCTTCTACCTTTCTC; Reverse: 5' GAGCAATTCGCCATTACTCTGAAGT). For all genotyping, PCR was run using an initial 2 minute 95⁰C denaturation followed by 35 cycles of 30 seconds at 95⁰C, 30 seconds at 55⁰C, 1 minute at 72⁰C, ending in a terminal extension of 7 minutes at 72⁰C.

Dosing of Mice and Isolation of microsomal protein and total RNA – Three ten-week-old male mice were used per treatment group per genotype. Mice were injected i.p. with 100 microliter doses of MeSO₄ (DMSO), TCDD (5 µg/kg in DMSO), PCN (10 mg/kg in DMSO) or TCPOBOP (3 mg/kg in DMSO) once daily for three days, and were sacrificed 24 hours after the final dose. Untreated male mice were sacrificed between ten and eleven weeks of age. Livers and colons of each mouse were collected and frozen on dry ice. The colon was first dissected longitudinally and rinsed with ice-cold 1.15% KCl prior to freezing. Organs were individually pulverized under liquid nitrogen and an aliquot of pulverized tissue was taken for either RNA or protein isolation. For microsomal preparation, tissue was homogenized using a motorized glass Teflon homogenizer in 5 volumes of ice-cold 1.15% KCl containing 1 mM PMSF. The homogenized solution was centrifuged at 3,000 x g for 10 minutes at 4⁰C, the supernatant was removed and centrifuged at 10,000 x g for 10 minutes at 4⁰C, and the resultant supernatant was centrifuged at 100,000 x g for 60 minutes at 4⁰C. The protein pellet was resuspended in 50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, and 1% protease inhibitor cocktail (Sigma-Aldrich). Protein

concentration was determined by the Bradford method. Total RNA was isolated using the TRIzol method (Invitrogen) and approximately 100 mg of pulverized tissue.

Analysis of Gene Expression Using RT-PCR – cDNA was synthesized from 1 µg of total RNA (isolated as described) using the iScript cDNA synthesis kit (BioRad) method. For each 20 µL PCR reaction, 1 µL of the reverse transcription reaction was used as template, along with 200 nM of forward and reverse mouse β-actin primers, 300 nM of UGT1A-specific primers (Strassburg et al., 1998; Strassburg et al., 1999) or 300 nM of Cyp1a1 (Galijatovic et al., 2004) or Cyp2b10 (Ueda et al., 2002) primers, 5 mM dNTP mix, and 1 unit of Taq (Denville) in proprietary buffer. PCR was performed as described (Chen et al., 2005). 10 µL of each reaction was visualized on a 1.8% agarose gel containing 1 µg/mL ethidium bromide. Quantitative real-time PCR was performed on a Stratagene MX4000 Multiplex QPCR. 1 µL of iScript cDNA product was used in 20 µL PCR reactions with Mesa Green qPCR MasterMix (Eurogentec, San Diego CA), and each sample was run in triplicate. Primers used were as follows. UGT1A1: F 5' aacaaggagctcatggcctcc, R 5' gttcgcaagattcgatggctg; UGT1A3: F 5' cagtggatgattctcagtc, R 5' ccatgttctccagaagcatta; UGT1A4: F 5' acgctgggctacactcaagg, R 5' tcattatgcagtagctccacacaa; UGT1A6: F 5' cttttcacagaccagccttac, R 5' tatccacatctctcttggagacag; UGT1A9: F 5' gaggaacattattatgccaccg, R 5' gcaacaaccaaattgatgtgtg, mouse carboxypeptidase-H (mCPH): F 5' atggtaaccccaccgtgt, R 5' ttcttgctgtctttggaactttgtc. UGT1A Ct values were normalized to mCPH.

Immunoblot Analysis – Protein analysis was performed by the Western Blot method using NuPAGE 4-12% Bis-Tris polyacrylamide gels (Invitrogen). 20 µg of protein in SDS loading buffer was heated at 70°C for 10 minutes before being run under denaturing conditions (50mM MOPS, 50mM Tris base pH 7.7, 0.1% SDS, 1mM EDTA). Protein was transferred to PVDF membranes using a semidry transfer system (Novex). The membranes were incubated in a Tris buffer solution (10mM Tris-HCL pH 7.4, 0.15 M NaCL, 0.05% Tween 20) containing 5% nonfat dry milk for 1 hour at room temperature, followed by overnight incubation at 4°C with a primary mouse anti-human UGT1A1 antibody (Chen et al, 2005). The membranes were washed with Tris buffer 6 x 10 minutes and then incubated with horseradish peroxide-conjugated anti-mouse antibodies for 1 hour at room temperature. The membranes were washed 4 x 10 minutes in Tris buffer. The horseradish peroxidase-conjugated protein was detected with the Western Lightning™ Chemiluminescent detection system (Perkin-Elmer) and a ChemiDoc XRS chemiluminescent camera (BioRad).

Results

To examine the contribution of PXR to the regulation of the *UGT1* gene locus, mice possessing no functional PXR (Xie et al., 2000a) were bred with mice expressing the entire human *UGT1* locus (Chen et al., 2005) to obtain a Tg-*UGT1*-PXRnull mouse model. These mice had no obvious phenotypic differences from wild type or Tg-*UGT1* mice, reproduced normally, and were of average weight and size.

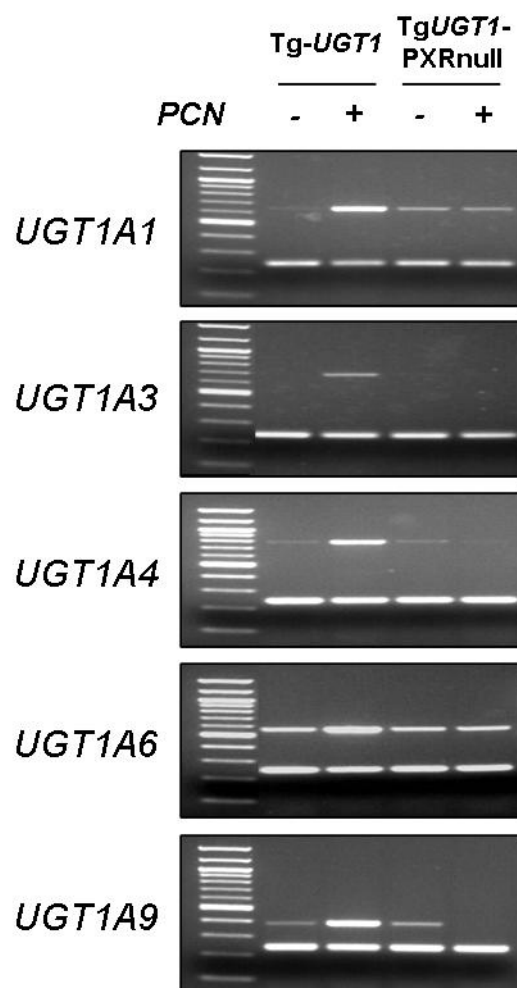


Figure 5-1. Induction of the UGT1A transcripts by PCN is lost in the Tg-UGT1-PXRnull livers. Livers isolated from 3 animals per treatment group were pooled and RNA was collected for RT-PCR. 1 μ g of RNA was used for reverse transcription, and PCR was run at 30 cycles using isoform-specific primers. Mouse β -actin primers were used as an internal control. Each PCR was run on a 1.8% agarose gel containing ethidium bromide.

Treatment of Tg-UGT1 and Tg-UGT1-PXRnull mice with the PXR ligand PCN resulted in upregulation of UGT1A1, UGT1A3, UGT1A4, UGT1A6, and UGT1A9 transcript in the livers of Tg-UGT1 but not Tg-UGT1-PXRnull mice, confirming the lack of functional PXR in these mice (Figure 5-1). Unexpectedly,

basal expression levels were higher in the PXR-null line for all isoforms examined. UGT1A1 transcript was 12-fold higher in the Tg-*UGT1*-PXRnull livers, UGT1A3 expression was elevated 10-fold, UGT1A6 was 6-fold higher, and UGT1A4 exhibited a very dramatic 400-fold increase compared to Tg-*UGT1* livers (Figure 5-2).

This result echoes the finding that basal levels of Cyp3a11, another PXR-inducible gene, are elevated in an independently-derived mouse line with targeted disruption of the PXR gene (Staudinger et al., 2001a). Interestingly, the same PXRnull mouse line used in these experiments was previously characterized (Xie et al., 2000a) and no such elevated basal Cyp3a11 was noted.

PXR has been implicated in cross-talk with the Constitutive Androstane Receptor (CAR) and the Aryl Hydrocarbon Receptor (AhR). Ligand-activated PXR can upregulate AhR (Xie and Evans, 2001) and CAR (Maglich et al., 2002), but unliganded or overexpressed human PXR suppresses CAR expression (Rosenfeld et al., 2003; Saini et al., 2005), suggesting that PXR controls these genes in a highly regulated manner. Moreover, in addition to PXR and CAR having overlapping target genes, CAR is able to bind and transactivate known PXR responsive elements and vice versa (Xie et al., 2000b). Experiments were undertaken to examine the influence of PXR on induction of the *UGT1* locus by CAR and AhR. (Figure 5-2) Tg-*UGT1* mice treated with the mouse CAR agonist TCPOBOP (Tzameli et al, 2000) exhibited elevated liver UGT1A1, UGT1A3, UGT1A4, UGT1A6, and cyp2b10. TCPOBOP-dependent induction of UGT1A1, 1A3, and 1A6 was also seen in Tg-*UGT1*-PXRnull livers, though at a lower magnitude than in the Tg-*UGT1* animals: the fold induction

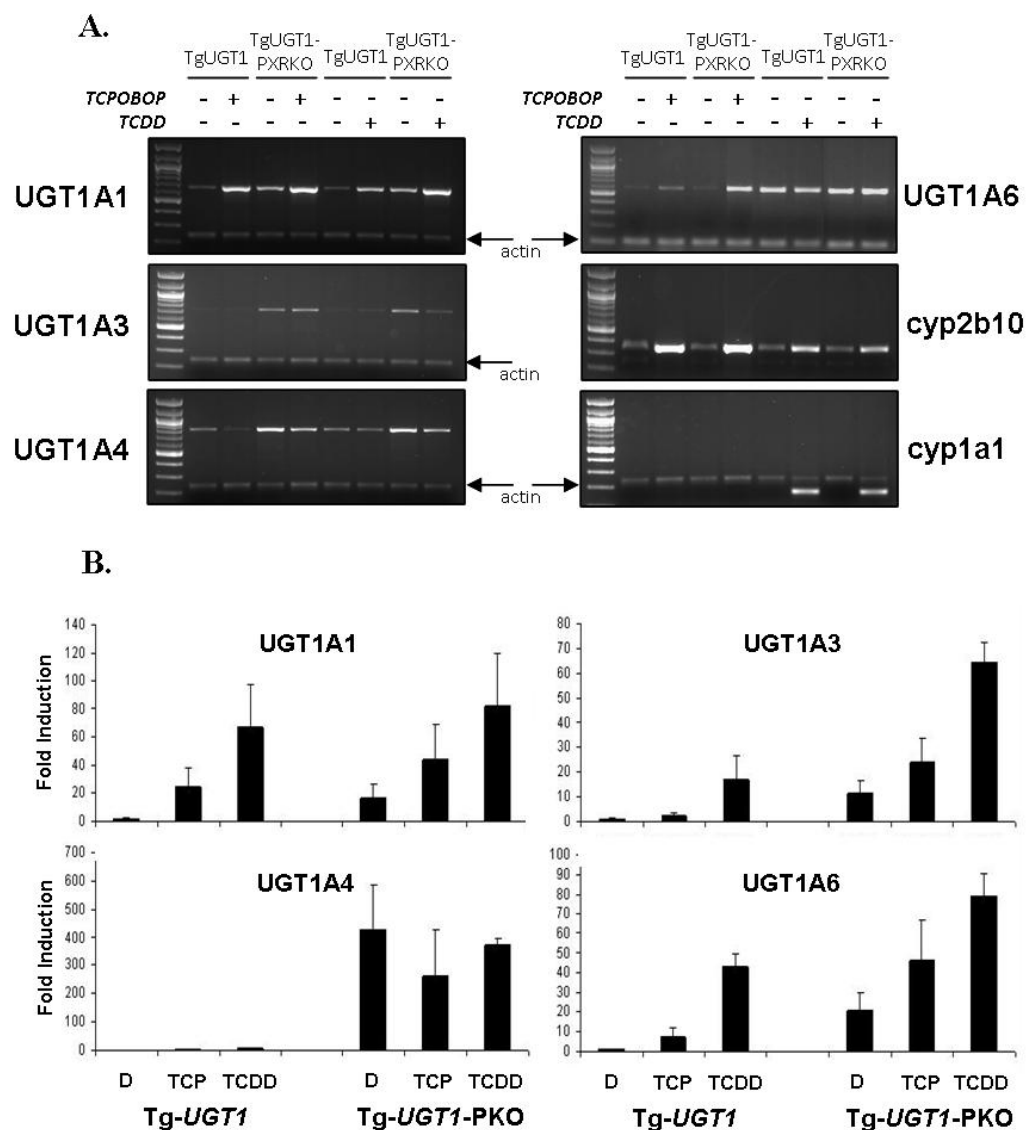


Figure 5-2. UGT1A Expression and Induction in Tg-UGT1 and Tg-UGT1-PXRnull Mouse Liver. Livers from 3 animals per treatment group were isolated and RNA was prepared as described. **A)** Liver RNA was pooled into treatment groups, 10 μ g of total RNA per liver. Reverse transcription was performed, followed by 30 cycles of PCR using isoform-specific primers. Mouse β -actin primers were used as an internal control. **B)** Quantitative RT-PCR was run using individual liver samples. Each sample was run in triplicate for UGT1A1, UGT1A3, UGT1A4, UGT1A6, and carboxypeptidase-H (CPH), a control gene. An average Ct value for each sample was obtained and was normalized to CPH. Fold change is expressed compared to the average of the Tg-UGT1 DMSO-treated samples.

of UGT1A1 in Tg-*UGT1* mice was 18-fold over DMSO compared to a 2.6-fold induction in the Tg-*UGT1*-PXRnull mice. Similarly, UGT1A3 induction was 2.3-fold over DMSO in Tg-*UGT1* mice compared to 2-fold in the Tg-*UGT1*-PXRnull line, and UGT1A6 induction decreased from 7.5-fold to 2.2-fold. This apparent decrease in CAR induction in the PXRnull background, however, does not necessarily reflect a diminished capacity for CAR to induce the genes and may rather exist because the basal levels of UGT1A1 and 1A3 in the Tg-*UGT1*-PXRnull livers are much higher than in the Tg-*UGT1* counterparts. Cyp2b10, a known CAR target, was induced equally well in the Tg-*UGT1*-PXRnull mice as in the Tg-*UGT1* mice. In this same way, UGT1A4 transcript increased a modest 3-fold with TCPOBOP administration in the Tg-*UGT1* mice compared to no appreciable increase upon treatment in the Tg-*UGT1*-PXRnull mice, but this lack of induction in the Tg-*UGT1*-PXRnull mice is likely due to the transcriptional machinery working at its upper limits to produce 400 copies of 1A4 transcript in these livers for every one copy in the Tg-*UGT1* livers, and not due to a decrease in the ability of CAR to stimulate gene transcription.

When Tg-*UGT1* mice were treated with the AhR agonist TCDD, liver UGT1A1, 1A3, 1A4, 1A6, and cyp1a1 were upregulated, a result that mirrors previous studies in the laboratory (Chen et al., 2005). The induction by TCDD was quantitatively observed to be 50-fold for UGT1A1, 15-fold for UGT1A3, 6.7-fold for UGT1A4, and 5.7-fold for UGT1A6. In the Tg-*UGT1*-PXRnull mice, basal and inducible expression was elevated compared to Tg-*UGT1*, similar to the results observed with TCPOBOP treatment. Induction by TCDD in the Tg-*UGT1*-PXRnull mice was also of a lesser magnitude than in the Tg-*UGT1*, with a 5-fold increase in

UGT1A1 transcript, a 6-fold increase in UGT1A3, a 1.7-fold increase in UGT1A6, and no increase in UGT1A4, though again this does not seem to be related to loss of AhR activity or capability to activate gene transcription, as evidenced by equally robust *cyp1a1* expression in both genetic backgrounds.

PXR is expressed in both humans and rodents in many different tissues where it functions as a xenobiotic sensor and homeostatic regulator of phase I, phase II, and phase III families of drug metabolizing enzymes (Blumberg et al., 1998; Kliewer SA et al., 1998; Xie et al., 2003; Vyhldal et al., 2004; Chen et al., 2005; Synold et al., 2001; Kast et al., 2002). Like many of the RXR-heterodimerizing nuclear receptors, PXR acts on its target genes in cooperation with sets of coactivator and corepressor proteins (Tsai and O'Malley, 1994) interacting through the AF2 and ID-2 domains, respectively (Danielian et al., 1992; Durand et al., 1994; Tone et al., 1994; Johnson et al., 2006; Wang et al., 2006; Watkins et al., 2003). As each tissue contains a unique complement and balance of transcriptional coactivators and corepressors, the ability for a nuclear receptor such as PXR to alter transcription may vary from tissue to tissue, and even from cell type to cell type. To determine whether PXR plays a tissue-specific role in the regulation of the *UGT1* locus, colon tissues from Tg-*UGT1* and Tg-*UGT1*-PXRnull mice that had been treated with TCPOBOP or TCDD were examined. In liver, the locus appeared to be suppressed by PXR in the absence of an activating ligand and this suppression was lifted in the PXR-null background, allowing for elevated basal levels of several isoforms. In the colon, however, this pattern of suppression was not maintained. (Figure 5-3) TCPOBOP treatment of Tg-

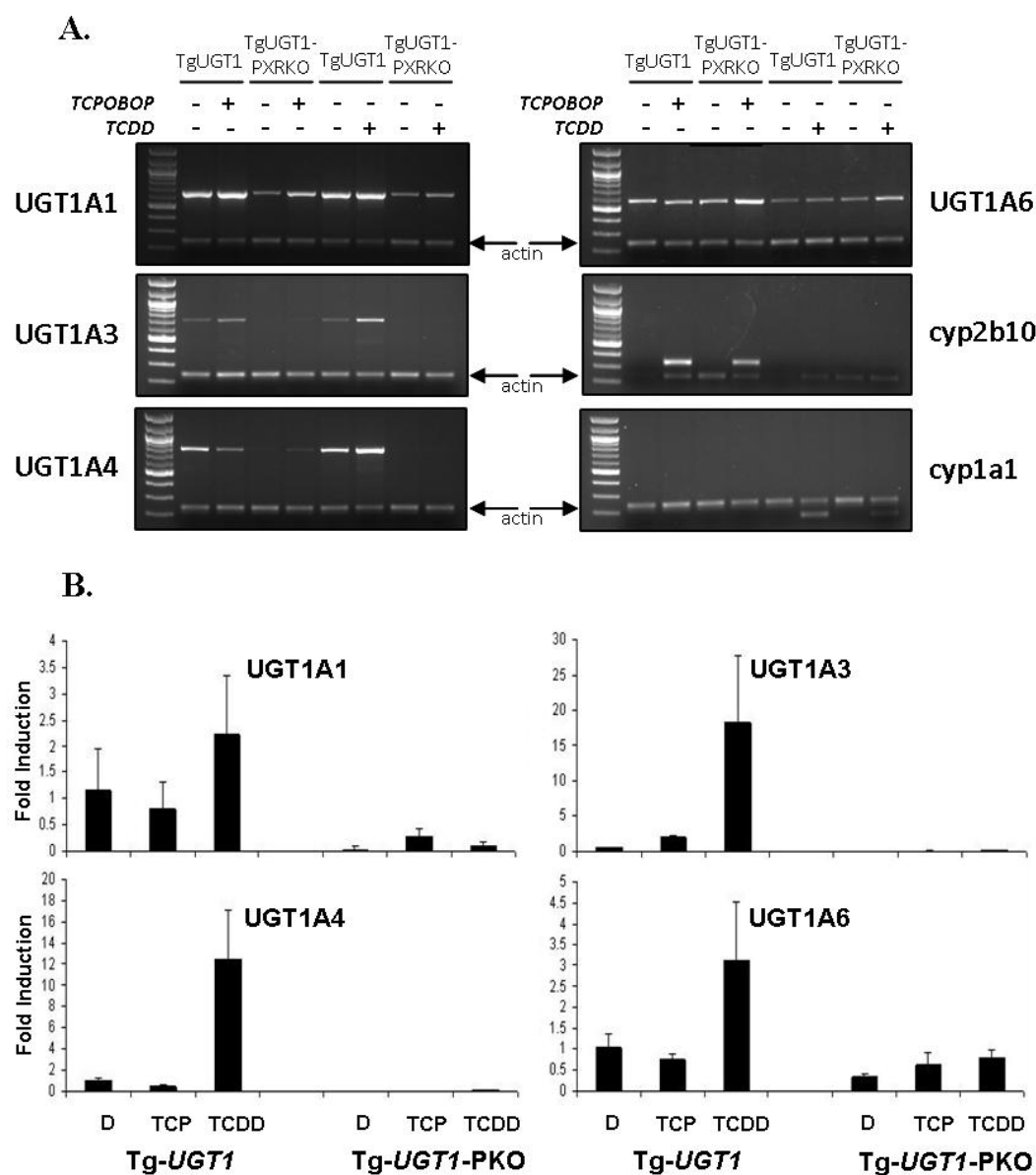


Figure 5-3. UGT1A Expression and Induction in Tg-UGT1 and Tg-UGT1-PXRnull Mouse Colon Tissue. The colon was excised from 3 animals per treatment group, sliced longitudinally, and rinsed with 1.15% KCl. RNA was prepared as described. **A)** RNA was pooled into treatment groups, 10 µg of total RNA per animal. Reverse transcription was performed, followed by 30 cycles of PCR using isoform-specific primers. Mouse β -actin primers were used as an internal control. **B)** Quantitative RT-PCR was run using individual samples. Each sample was run in triplicate for UGT1A1, UGT1A3, UGT1A4, UGT1A6, and carboxypeptidase-H (CPH), a control gene. An average Ct value for each sample was obtained and was normalized to CPH. Fold change is expressed compared to the average of the Tg-UGT1 DMSO-treated samples.

UGT1 mice showed little to no induction of the locus, which is not an unexpected result given the very low levels of CAR in the large intestine. Conversely, TCDD treatment resulted in induction of UGT1A1, UGT1A3, UGT1A4, and UGT1A6 transcript. In the Tg-*UGT1*-PXRnull mice, however, UGT1 transcript levels were very low in both untreated and treated tissues. In vehicle-treated Tg-*UGT1*-PXRnull colon tissue, UGT1A6 was 3-fold lower than in Tg-*UGT1*, UGT1A1 was 35-fold lower, and UGT1A3 and 1A4 were not detected. Though treatment with either TCPOBOP or TCDD elicited a small amount of induction, these values were well below the basal expression level in mice expressing PXR, the implying a role for PXR in supporting uninduced and induced expression in the colon that differs from the suppressive role it appears to play in the liver. This differential expression is maintained at the protein level (Figure 5-4).

PXR is a conserved nuclear receptor that exhibits high homology within mammalian species (Jones et al., 2000). The DNA binding domains are most highly conserved, sharing upwards of 95% homology among mammalian species, compared to 77% homology between the ligand binding domains of mouse and human PXR (Kliewer et al., 2002). The divergence in LBD identity corresponds with a divergence in ligand specificity: Pregnenolone-16 α -carbonitrile (PCN) and dexamethazone are a very good activator of mouse PXR but have little activity on human PXR, while the drugs rifampicin and clotrimazole activate the human but not mouse receptor (Blumberg et al., 1998). Many endogenous steroid ligands for which

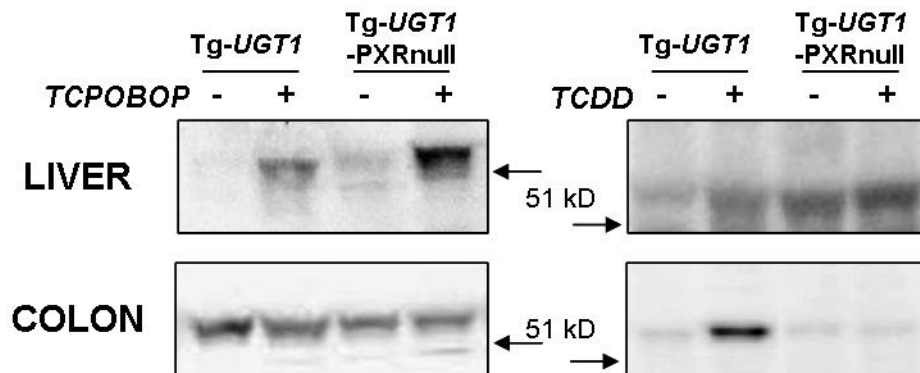


Figure 5-4. UGT1A1 Protein Expression in Tg-UGT1 and Tg-UGT1-PXRnull mouse tissues. Liver and colon tissues from 3 animals per treatment group were isolated and microsomes were prepared as described. 50 μ g of microsomal protein per sample was pooled into treatment groups and 20 μ g of each pool was run under denaturing conditions and transferred to nitrocellulose membranes. Protein was detected using a mouse anti-human UGT1A1 antibody that does not cross-react with mouse Ugt protein (Chen et al, 2005).

the Pregnane X Receptor was named also activate in a species-dependent way (Moore et al., 2000; Jones et al., 2000; Kliewer SA et al., 1998; Blumberg et al., 1998). To examine the ability of human PXR to activate the human *UGT1* locus, a humanized and constitutively activated PXR mouse model was obtained as a generous gift from Dr. Ron Evans (Salk Institute, CA). This mouse possesses human PXR cDNA fused with a VP16 activation domain which renders the PXR protein constitutively active in the absence of ligand. Expression of the cDNA is under control of an albumin promoter, thus expression is liver-predominant. Tg-UGT1, Tg-UGT1-PXRnull, and Tg-UGT1-hVPPXR mouse livers were analyzed for UGT1 expression. VPPXR is able to dramatically upregulate UGT1A1 protein expression (Figure 5-5B) as well as 1A1, 1A3, 1A4, 1A6, and 1A9 transcript (Figure 5-5A), a pattern which is similar to the regulation of the human *UGT1* genes by mouse PXR in Tg-UGT1 mice (Chen et

al., 2005). hPXR responsive elements have been previously identified in UGT1A1 and 1A6 upstream regions (Vyhlidal et al., 2004; Xie et al., 2003).

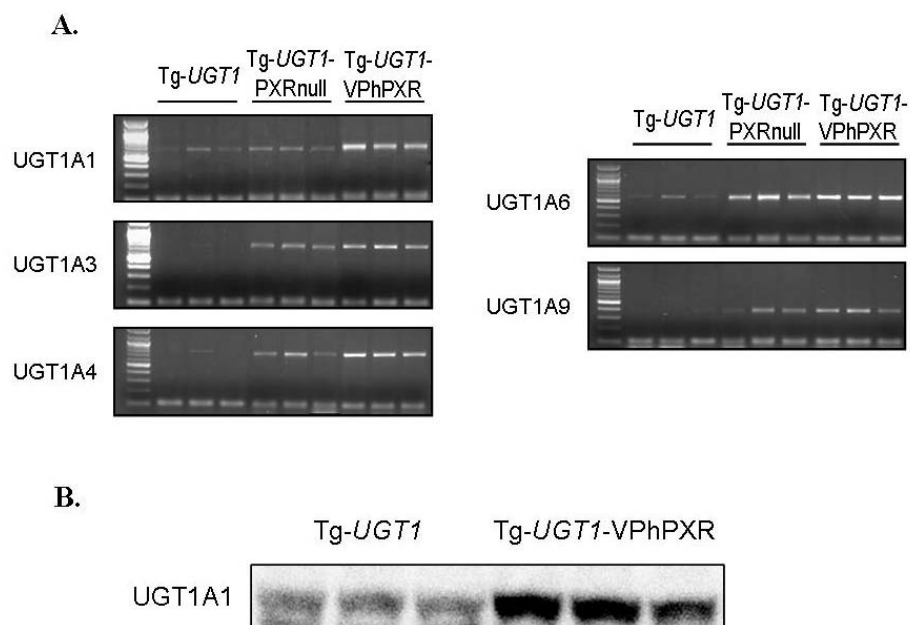


Figure 5-5. UGT1A Expression in Tg-UGT1, Tg-UGT1-PXRnull, and Tg-UGT1-VPhPXR Livers. Livers from 3 animals per genetic strain were isolated. A) RNA was prepared from individual samples and RT-PCR was performed at 30 cycles using isoform-specific primers. Mouse β -actin was used as an internal control. B) Microsomal protein was prepared from individual samples and 20 μ g of protein per sample was run under denaturing conditions on a 4-12% Bis-Tris polyacrylamide gel. Protein was transferred to a nitrocellulose membrane and was detected using a human-specific UGT1A1 antibody (Chen et al, 2005).

Discussion

Among the nuclear receptors, PXR is most commonly associated with control of xenobiotic metabolism, and activation of phase I, II, and III genes by PXR ligands has been well characterized. In this study we demonstrate that PXR plays a central role in control of the human *UGT1* genes, not only through transcriptional activation,

but also as a basal suppressor and tissue-specific regulator. Mice expressing no functional PXR were bred into a line of mice containing the entire human *UGT1* gene locus. These Tg-*UGT1* mice have been previously characterized by us and express the *UGT1* genes in a tissue-specific and inducible manner, with all nine expressed isoforms being upregulated by PCN in either the liver, small intestine, or large intestine (Chen et al., 2005). After PCN treatment, Tg-*UGT1*-PXRnull mice do not demonstrate induction of the *UGT1* genes, indicating that PXR in this line is in fact not functional and confirming that PCN induction of the *UGT1* locus in Tg-*UGT1* mice is mediated by PXR (Figure 5-1). It was observed that uninduced levels of the UGT1 transcripts were higher in the Tg-*UGT1*-PXRnull livers when compared to the Tg-*UGT1* livers. This result was quantified using quantitative RT-PCR, and the increase in basal expression ranged from moderate (UGT1A6, 6-fold) to dramatic (UGT1A4, 400-fold). This phenomenon is not unprecedented: Staudinger et al reported in 2001 that a PXR-null mouse derived in their laboratory exhibited elevated mouse liver cyp3a11 RNA compared to wild type levels (Staudinger et al., 2001a). Additionally, higher basal expression of several mouse drug metabolism genes, including Cyp2b10, Ugt1a1, Gsta2/4, Mrp2, and Oatp4 has been demonstrated in the same PXRnull line used in the development of our Tg-*UGT1*-PXRnull mice. (Saini et al., 2005; Rosenfeld et al., 2003). This study, however, is the first description of this regulatory pattern in human drug metabolism genes.

Because CAR, AhR, and PXR have been linked to regulation of many of the same genes (Maglich et al., 2002; Kohle and Bock, 2008; Kretschmer and Baldwin, 2005), can regulate one another (Xie and Evans, 2001; Maglich et al., 2002; Rosenfeld

et al., 2003;Patel et al., 2007), and PXR and CAR can bind to the same DNA sequences (Xie et al., 2000b;Xie W and Evans RM, 2001;Konno et al., 2008), the influence of PXR on regulation of the UGT1 locus by CAR and AhR was examined using the Tg-*UGT1* and Tg-*UGT1*-PXRnull mouse lines (Figures 5-2 and 5-4). Treatment of the mice with the mouse CAR agonist TCPOBOP resulted in induction of UGT1A1 transcript and protein in the liver. Given that PXR can control expression of CAR, it was predicted that loss of PXR may negatively affect the expression of genes under control of CAR. However, in mice with no functional PXR (Tg-*UGT1*-PXRnull), the CAR-responsive mouse gene *cyp2b10* was induced at the same magnitude as in the Tg-*UGT1* mice. The human *UGT1* genes were also induced in the PXRnull background, though the fold induction over DMSO-treated mice was significantly lower than in Tg-*UGT1*. It was observed that CAR was able to induce *cyp2b10* normally, therefore this is not likely a result of modulation of CAR activity but instead resultant from abnormally high basal transcriptional levels.

Administration of the AhR agonist TCDD yielded similar findings in that UGT1A1, 1A3, 1A4, 1A6, and *cyp1a1* were induced by TCDD in both Tg-*UGT1* and Tg-*UGT1*-PXRnull livers. The order of magnitude of the induction was decreased in the PXRnull background, but *cyp1a1*, a gene under control of the AhR, was also induced equally well in both mouse strains, demonstrating that loss of PXR did not affect the ability of the nuclear receptor to induce its target genes. Because activated PXR can upregulate both CAR and AhR, it was surprising that loss of PXR had no effect on the nuclear receptors and appeared to affect the *UGT1* gene locus directly.

The elevated basal expression and induction of the *UGT1* genes in the PXRnull background suggest an important and heretofore uncharacterized role for PXR as a basal suppressor of the human *UGT1* locus. Chen and Evans and Horlein et al first reported in 1995 that cofactors interacting with unliganded nuclear receptors of the thyroid hormone and retinoid receptor subfamily, which we now understand to include PXR, CAR, VDR, LXR, and PPARs, acted as repressors. These cofactors were termed N-Cor (nuclear receptor co-repressor) (Horlein et al., 1995) and SMRT (silencing mediator for retinoid and thyroid-hormone receptors) (Chen and Evans, 1995). The first report of PXR-SMRT interaction and SMRT-mediated repression of PXR activity came in 2001 (Synold et al., 2001), and in recent years the interaction domain has been mapped to the AF-2 motif on the PXR protein (Wang et al., 2006;Johnson et al., 2006). Repression is released upon ligand binding, causing dissociation of SMRT and recruitment of coactivators and transcriptional machinery (Kliewer SA et al., 1998;Yao et al., 1996;Kamei et al., 1996;Spencer et al., 1997;Jenster et al., 1997;Chen et al., 1999;Schurter et al., 2001). This unliganded-repression model for PXR regulation has not previously been associated with *UGT1* genes with the exception of the previous chapter in this dissertation; the studies reported here provide in vivo evidence that PXR may interact with the gene locus in this manner.

PXR is expressed throughout the body in organs including the liver, intestines, colon, kidney, stomach, lung, and in the uterus, ovaries, and placenta (Kliewer SA et al., 1998;Lehmann et al., 1998;Bertilsson et al., 1998;Blumberg et al., 1998;Masuyama H et al., 2001). Given its wide range of ligands, the function of

PXR is likely tissue-dependent: in the liver, it serves as a xenobiotic and bile acid sensor, whereas in the ovaries it likely acts as a hormonal sensor to regulate hormone turnover and homeostasis. Thus, it was hypothesized that its role regarding control of the *UGT1* genes may be tissue-specific as well. Colon tissues from Tg-*UGT1* and Tg-*UGT1*-PXRnull mice were analyzed for UGT1 transcript levels (Figure 5-3). As expected, untreated Tg-*UGT1* mice expressed UGT1A1, 1A3, 1A4, and 1A6. TCPOBOP treatment elicited a small induction of UGT1A3 but little induction of the other transcripts. Although CAR is highly expressed in the liver, there are only low levels of CAR in the mouse colon, therefore it is not surprising that induction of the UGT1As by TCPOBOP was limited. TCDD treatment, however, resulted in robust induction of UGT1A1, UGT1A3, UGT1A4, and UGT1A6 in the Tg-*UGT1* mouse colon tissues. Tg-*UGT1*-PXRnull mice, however, had little to no expression of the same genes, and induction by TCDD was lost in the PXRnull background. The role for PXR in the colon appears to be quite different from the liver. Whereas PXR appears to actively suppress transcription of the human *UGT1* genes in the liver, expression and inducibility in the colon rely on PXR, and loss of PXR effectively shuts down the gene locus, making it resistant to induction through other nuclear receptors.

It was next examined whether introduction of activated human PXR into the Tg-*UGT1*-PXRnull animals would upregulate the *UGT1* genes in a manner consistent with that observed in Tg-*UGT1* mice treated with mPXR agonists. The human and mouse PXR DNA Binding Domains (DBD) are ~95% homologous and a constitutively active hPXR has previously been shown to upregulate many known

mPXR target genes (Xie et al., 2000a). This mouse line contains human PXR cDNA fused at the N-terminus with a VP16 viral protein activation domain which renders the unliganded receptor constitutively active. Use of a constitutively activated PXR avoids the need for chemical activation of the receptor, which harbors its own complications such as secondary drug effects. It also circumvents potential species-specific interactions between the human receptor's AF2 and domain and coactivator or corepressor proteins essential for proper receptor transactivation. The VP-hPXR cDNA construct is under the control of an albumin promoter which yields liver-specific expression of the activated human receptor. Mice possessing this construct were obtained and bred into Tg-*UGT1*-PXRnull mice to create a Tg-*UGT1*-VPhPXR line that expresses the *UGT1* locus and *alb*-VP-hPXR in an mPXR-null background. Analysis of mouse livers from this line show constitutive upregulation of the *UGT1* battery of liver-expressed genes (*UGT1A1*, *1A3*, *1A4*, *1A6*, and *1A9*) compared to the Tg-*UGT1*-PXRnull livers (Figure 5-5). *UGT1A1* protein was also dramatically induced. These data confirm that human PXR upregulates the human *UGT1* genes in a pattern similar to the mouse PXR, and strengthen confidence in a mouse model for PXR action on drug metabolism.

Humanized *alb*-VP-hPXR mice have been previously characterized and exhibit negative physiological effects such as enlarged liver, liver toxicity, and growth retardation (Xie et al., 2000a). This provides an insight into the importance of basal suppression of the *UGT1* locus and other drug metabolism genes. Uncontrolled activation of these genes clearly lead to a toxic phenotype, perhaps due in part to greatly increased phase I biotransformation of endogenous and exogenous

compounds into toxic intermediaries. Thus, PXR's action as an unliganded suppressor is likely a protective measure, ensuring precisely regulated expression of its target genes and subsequent tight homeostatic maintenance of many biological systems essential for proper health. This is highlighted by the results of this study, in which PXR is shown to maintain a suppressive role toward *UGT1* expression in the liver. The differential role that PXR plays in the colon, where PXR expression underlies basic transcription of many *UGT1* genes, is likely related to the different roles the organs play in drug metabolism. These results highlight the importance of PXR as a master regulator of the *UGT1* genes and provide insight into the breadth of PXR's control over drug metabolism.

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CHAPTER 6
CONCLUSIONS

At the outset of my thesis work, it was known that the UGTs were targets for regulation by the nuclear receptors. Induction studies in immortalized or hepatocyte cell culture had demonstrated that UGT1A1, in particular, was upregulated by treatment with xenochemicals that were known inducers of AhR, PXR, and CAR, and with the identification of DNA response elements for these receptors (Yueh et al., 2003; Xie W et al., 2003; Sugatani et al., 2001) came a better understanding of the role that xenobiotics play in control of phase II drug metabolism. My studies have focused on characterizing NR control of the *UGT1* genes, with a particular focus on UGT1A4.

UGT1A1 Induction by PPAR α

Although human hepatocyte cultures retain some expression and induction of the liver-expressed UGT1As, namely UGT1A1, 1A3, 1A4, 1A6, and 1A9, it was surprising to observe a total loss of expression or induction of UGT1A3 and 1A4 in Tg-*UGT1* hepatocytes. The observation that this downregulation occurs within hours of plating suggests that this is an artifact of the attachment and microtubule extension process. That HepG2 cells, which also do not express 1A3, 1A4, or several other proteins important in drug metabolism such as CYP3A4, PXR, and CAR, are capable of expression and induction in a 3-D culture system or when treated with microtubule stabilizing drugs (Horiuchi et al., 2009) lends weight to this hypothesis. Implementation of these techniques in future HepG2 or Tg-*UGT1* hepatocyte culture may allow for future induction studies of others UGTs.

Although HepG2 cells do express UGT1A1, induction by the type II NRs requires transfection and overexpression. Development of a HepG2 cell line in which human PPAR α cDNA is stably integrated into the genome (hPPAR α -HepG2) (Hsu et al., 2001) provided a cell culture system in which to examine PPAR α interaction with the *UGT1A1* promoter and enhancer sequence. Through use of *UGT1A1* promoter deletion and enhancer constructs, the PPAR α -responsive element was mapped to a DR1 sequence within the PBREM, and this sequence was further confirmed to be PPAR α -responsive through use of a concatenated-DR1 enhancer construct. It is perhaps not surprising PPAR response was localized to this region, as nearly all of the nuclear receptor elements have been mapped to this 300 bp region. The presence of such a regulatory “nucleus” is unique among the *UGT1* genes, and an evolutionary advantage for its existence is not completely clear. The proximity of these elements is likely to introduce steric limitations; that is, nuclear receptors may have to compete for DNA binding. Functionally, however, this could be advantageous: by setting a limit on the gene’s inducibility, processes such as bilirubin clearance are kept within homeostatic parameters. Having numerous closely-spaced response elements may also allow for coordinate activation by multiple NRs and enhanced recruitment of coactivators and transcriptional machinery. The unique role of UGT1A1 as the sole enzyme controlling a detoxification pathway essential to human health may have resulted in formation of this response element core to ensure tight regulation of the gene.

The activation of several *UGT1* genes not associated with fibrate metabolism or the lipid metabolic pathway by PPAR α demonstrates the promiscuity of NR

activation by xenobiotics. It also shows, however, the capacity for enhanced detoxification response after xenobiotic insult. By understanding the scope of the PPAR α -activated battery of genes, it is possible to better predict adverse drug interactions by understanding the potential for accelerated clearance of UGT1A-metabolized drugs.

Induction of UGT1A4 by the Nuclear Receptors

Despite the fact that UGT1A4 plays an important role in the metabolism of several commonly used drugs and it is known to be induced in vivo by PXR, AhR, and PPAR, little is known about the elements that govern its regulation by these nuclear receptors. This is due, in large part, to the lack of an appropriate in vitro model system with which to study UGT1A4 induction. The colorectal adenocarcinoma cell line, LS180, was identified as a 1A4-expressing and 1A4-inducible cell culture line, and this line was used to identify several nuclear receptor response sites within the regulatory region.

Unlike UGT1A1, UGT1A4 NR response sites were scattered throughout the 4.96 kb regulatory region, with putative RE at -4790 (DR1), -3230 (XRE), -2672 (DR3), -2159 (DR3), -366 (DR1), and -232 (DR3). WY induction was isolated to a 150 bp sequence that encompassed the -366 DR1 element and mutational analysis confirmed this as the primary PPRE on the *UGT1A4* promoter. The primary AhR-responsive element (XRE) was identified ~3.2 kb upstream from the transcription start site; deletion past this element or mutation of its core consensus site abolished induction by the AhR ligand TCDD. Interestingly, a recent study examined just the proximal 300 bp of the *UGT1A4* promoter and found two XRE within this sequence

to confer inducibility by TCDD (Erichsen et al., 2008). As the experiments presented in this dissertation were performed prior to the publication of this referenced study, AhR-mediated induction of constructs smaller than 1kb (P4) was not investigated.

A putative PXR response element (PXRE) (DR3) at -232 was investigated through promoter deletion and mutational analysis. Deletion past the DR3 element or mutation of residues within it not only abolished PXR-mediated induction, but also resulted in a large increase in basal luciferase activity. Using freshly prepared plasmid DNA and controlling for transfection efficiency and DNA quality through use of a dual luciferase reporter system, uninduced luciferase activity of these constructs was found to be 13-17-fold higher than on longer constructs containing a non-mutant DR3. These data suggest that this DR3 element and the proteins interacting with it actively suppress transcriptional activity of the *UGT1A4* gene. Further experiments must be performed to determine whether these proteins do, in fact, include PXR. These experiments, including Chromatin-Immunoprecipitation (ChIP) analysis, could confirm the interaction of PXR, corepressors, and the DR3 nucleotide sequence. It is known that PXR can interact simultaneously with DNA response sequences and corepressors such as SHP in the absence of ligand (Synold et al., 2001;Ourlin et al., 2003;Hariparsad et al., 2009), however, this has not previously been demonstrated for the UGTs. This mechanism of corepression also appears to be specific to PXR; corepressor binding to other related nuclear receptors, including ER, AR, AhR/Arnt, LXR, and RXR, and FXR, exerts its suppressive effects through inhibition of the dimer-corepressor complex's interactions with DNA response elements (Seol et al., 1998;Klinge et al., 2001;Gobinet et al., 2001;Brendel et al.,

2002;Goodwin et al., 2000;Lee et al., 2000). The evidence in this study points toward this model of PXR suppression of UGT1A4 transcription, and represents the first molecular evidence that PXR may act in a suppressive manner toward the *UGT1* genes. This may also offer an explanation for the conflicting results between the XRE mapped in this study and those found in Erichsen *et al*, in which two proximal XRE were identified as the primary AhR-responsive elements. In the absence of the suppressive PXR element, the constructs used in Erichsen *et al* were susceptible to induction by TCDD.

In this referenced study, *1A4* promoter constructs were transfected into HepG2 cells, in which it has been demonstrated by myself and others that UGT1A4 transcript or longer *UGT1A4* promoter constructs are not effectively expressed or induced by PXR or AhR. Yet very short *1A4* promoter constructs appear to be readily induced by TCDD, which implies a suppressive effect on longer constructs or the native promoter. It has previously been assumed that the reason for loss of basal or inducible UGT1A4 expression in HepG2 cells was due to this cell line's lack of functional type II nuclear receptors, including PXR: transfection of constructs overexpressing PXR into HepG2 cells was sufficient to restore induction of UGT1A3 and 1A4 (Gardner-Stephen et al., 2004). It is therefore counterintuitive that in these cells that have little to no capacity for PXR transactivation, basal 1A4 transcription is suppressed. PXR functionality, particularly in the HepG2 line, has been typically ascertained through transactivation studies in which the ability for the nuclear receptor to recruit transcriptional machinery and induce gene expression is assessed. These studies do not, however, account for "functional" suppression of target genes.

PXR is expressed at both the mRNA and protein levels in HepG2 cells; the lack of transactivation has been previously hypothesized to be due to accelerated protein degradation or inhibition of nuclear translocation due to microtubule disarray (Horiuchi et al., 2009; Dvorak et al., 2005). Recent studies, however, have revealed that splice variants of hPXR have differential transactivation potential. Wild-type hPXR (T1) and variant T2 which has a 39-amino acid N-terminal insertion exhibit full transactivation potential, while a variant containing a 37-amino acid deletion (T3) in the ligand binding domain can act as a dominant-negative, reducing or fully inhibiting transactivation by WT or T2 constructs (Lin et al., 2009). All three PXR splice variants are expressed in HepG2 cells (Gardner-Stephen et al., 2004), and repression of the *UGT1A4* gene or extended promoter constructs by this T3 variant may account for the “functional” repression observed. Another possible explanation for the active repression of the gene may be the presence of an inhibitory ligand. In vivo, an endogenous inhibitory ligand has not yet been identified; however, several classes of xenochemicals have been shown to interact with PXR and cause active suppression of drug metabolism genes (Synold et al., 2001; Lin et al., 2009; Huang et al., 2007). The presence of non-human and non-native ligands in cell culture media may result in the introduction of inhibitory compounds that can result in the silencing of specific drug metabolism genes by PXR in HepG2 cells. That this phenomenon is not observed in LS180 cells likely owes to the same tissue- and cell-specific factors that govern the differential expression patterns observed throughout the human body and Tg-*UGT1* mice.

Hormonal Regulation of the Human *UGT1* Locus during Estrus

Changes in drug metabolism profiles have been associated with pregnancy and lactation both clinically in humans and in the Tg-*UGT1* mice. The development of these mice enabled a full characterization of the extent of hormonal regulation, which was shown to involve several UGT1A isoenzymes and extensive upregulation in the mid-to-late stages of pregnancy. These mice were also used to characterize human *UGT1* changes throughout the mouse estrus cycle. Expression of the UGT1 transcripts varied dramatically by cycle phase, with as much as 100-fold changes in transcript levels within a 24 hour period. Downregulation was so significant during Diestrus II that UGT1A1, 1A3, and 1A4 transcript levels as assessed by quantitative RT-PCR approached the lower limit of detection by the Multiplex QPCR instrument. This study represents the first evidence for regulation of human UGT regulation during the reproductive cycle.

Although the hormonal changes that occur during estrus or the human menstrual cycle are much less dynamic than those that occur in pregnancy, these resultant alterations in expression could have important clinical implications. Circadian signaling is associated with highly transient 0.5- to 2-fold changes in drug metabolizing gene transcription, and yet these changes are sufficient to induce temporal toxic reactions to drugs such as cancer chemotherapeutics and high-dose acetaminophen (Zhang et al., 2009). Should the altered expression levels observed during estrus translate to even small changes during the human menstrual cycle, these fluxuations would still be clinically relevant, particularly in women taking drugs with low therapeutic indices.

It has become clear in the last few years that PXR plays a critical role in regulation of drug metabolism and the UGT1A family in particular. With its unusually extensive tissue expression pattern, the potential for active suppression via inhibitory ligands or splice variants, the atypical structural features that distinguish it from other NRs in its family, an unusually flexible ligand binding pocket that allows for association with ligands of many shapes and sizes, and its ability to regulate each *UGT1* gene as well as several phase I and III metabolism genes, PXR has emerged as a master regulator of drug metabolism. The regulation of the *UGT1* locus during pregnancy and estrus, combined with known activation of the receptor by the circulating hormones involved in these reproductive processes, led to PXR being the hypothesized mediator of hormonal control of the locus. This hypothesis was tested by assessing *UGT1* regulation during estrus in Tg-*UGT1*-PXRnull mice. Loss of PXR was predicted to result in a loss of *UGT1* regulation during estrus; however, the regulatory pattern was largely intact in the PXRnull background, clearly demonstrating that PXR was not essential to hormonal regulation. Additional experiments performed in the laboratory ascertained that the dramatic upregulation observed during pregnancy was also intact in the Tg-*UGT1*-PXRnull mice.

Although the basic pattern of regulation involving a Diestrus II-associated loss of UGT1 transcript was observed in the Tg-*UGT1*-PXRnull mice, there were notable differences in the regulatory patterns in the two mouse lines. Transcript levels during Diestrus II in the Tg-*UGT1*-PXRnull mice were several-fold lower than in the Tg-*UGT1* mice, and UGT1A3 and 1A4 QPCR values were comparable to those obtained from negative controls containing no cDNA template. UGT1 transcript levels were

also much higher during the Proestrus phase in the Tg-*UGT1*-PXRnull mice. Therefore, loss of PXR was not without impact on the regulation of these genes.

PXR is a Tissue-Specific Master Regulator of the Human *UGT1* Locus

Although PXR was not found to mediate hormonal control of the *UGT1* locus during estrus or pregnancy, it became clear that loss of PXR does still have a significant effect on expression. Further characterization of the Tg-*UGT1*-PXRnull mice led to the observation that basal transcriptional levels of the liver-expressed human *UGT1* isoforms were elevated in the PXRnull background. UGT1A4, in particular, exhibited extremely high basal transcription in the livers of these mice. Although dysregulation of several mouse phase I and phase III genes as well as mouse *Ugt1a1* has been observed in PXRnull mice, this report is the first to identify such in vivo dysregulation of human drug metabolism genes. It was plausible to assume that these changes were due to PXRnull-related alterations in signaling by other nuclear receptors, particularly those such as CAR and AhR that are known to be regulated by PXR and cross-activate its target genes. These receptors, however, were found to activate mouse Cyp target genes normally, indicating that loss of PXR had little to no overall impact on the capacity for induction by CAR and AhR. This implies that the observed elevation in basal transcription is caused by a loss of PXR function directly, through loss of an important regulatory role that the receptor plays. It was reported that in cell culture, ligand-free PXR can suppress transcription of the liver phase III transporter MRP2 (Saini et al., 2005). MRP2 is a target gene for both CAR and PXR, and in vitro studies showed that unliganded PXR prevented

coactivator recruitment by unliganded constitutively active CAR. Addition of a CAR ligand, however, restored CAR's ability to recruit coactivators. This may provide a molecular clue as to the role PXR plays in suppression of the *UGT1* locus in the liver, acting as a gatekeeper for transcriptional activity. Unliganded PXR inhibits transactivation by other NRs and allows for moderate basal transcriptional activity. Upon NR activation, PXR suppression is released and the target genes are activated. This may occur through competition for NR binding, or it may result from an overwhelming recruitment of coactivators via the activated NR.

This model for PXR action explains not only the changes in basal or induced transcriptional activity seen in these male Tg-*UGT1*-PXRnull mice, but also the differences in *UGT1* regulation in the two mouse lines during female mouse estrus (Chapter 4). When the male mice were treated with CAR or AhR ligands, *UGT1* transcription was induced in both lines, with induction in the PXRnull background resulting in significantly higher transcriptional activity. In the female mice, the basic pattern of regulation was maintained, with a large drop in UGT1 transcripts during Diestrus II. In the Tg-*UGT1*-PXRnull mice, these Diestrus II levels were lower, and Proestrus levels were significantly higher than in Tg-*UGT1*, demonstrating that loss of PXR correlates with the loss of a regulatory factor that allows for basal transcriptional activity and yet prevents unmitigated upregulation. A possible physiological reason for this role is observed in mice that overexpress a constitutively active hPXR. These mice develop liver toxicity and have shortened lifespans due to elevated levels of drug metabolizing enzymes. In Tg-*UGT1*-VPhPXR mice, UGT1A1 protein levels were dramatically elevated, and all five liver UGT1

transcripts were upregulated. The resulting abnormally high activity toward UGT1A substrates can result in increased clearance of endogenous compounds and subsequent dysregulation of any number of homeostatic processes. Therefore it is clear that controlled, moderate expression of these genes is critical for maintenance of health and prevention of toxicity. By acting as a master regulator of *UGT1* gene expression, PXR can ensure proper basal transcriptional activity while also allowing for transient increases upon xenobiotic assault to facilitate in clearance of these exogenous compounds.

Upon examination of other organs important to drug metabolism, a very different result from loss of PXR was noted. In the colon of PXRnull mice, very little UGT1 transcriptional activity was observed, and induction by AhR and CAR was very minimal. PXR therefore appears to control gene expression in a tissue-specific manner, and in the colon it is essential to maintain proper expression and inducibility of the *UGT1* genes. As each tissue expresses its own complement of signal transduction pathways, transcription factors, and coactivator and corepressor proteins, it is possible that the liver-expressed factors that facilitate unliganded PXR's suppressive function are not expressed in the colon. It is also possible that an endogenous PXR agonist exists in this tissue and enables PXR to constitutively activate gene transcription.

Given this tissue-specific role, it is paradoxical that in the *UGT1A4* promoter studies (Chapter 3), a suppressive role for PXR was noted not in the liver hepatocyte-derived HepG2 cells but rather in the colon-derived LS180 cells. However, immortalized cell lines are not always a reliable predictor of in vivo signaling. These

immortalized cell lines, including HepG2 and LS180, are often derived from cancer cells, whose signaling pathways have been altered enough to escape apoptosis and enable continuous growth and division; the differences between signaling in the cells and in the tissue from which they were isolated can be vast. Tissue-specific signaling also relies on paracrine and endocrine signals that are lost in an in vitro culture system. Thus, it is perhaps not surprising that the in vivo regulatory effects cannot be recapitulated in a cell culture system. Additionally, PXR can interact with an unusually broad array of compounds, and components of the cell culture medium may cause nonphysiological signaling as a result. This disparity reinforces the importance of in vivo model systems when investigating pathways critical for human health.

Final Remarks

Although PXR is the most recently characterized of the RXR-heterodimerizing type II receptors, in the eleven years since its initial characterization much has been learned about its unique role within the adopted orphan family. Studies published in just the last few years have revealed crucial facts about the structure of PXR and its interactions with coactivators and corepressors, providing strong evidence that PXR is capable of acting as an unliganded repressor. The work performed during my dissertation studies has revealed not only an in vivo demonstration of this unique regulatory function, but also an in vitro promoter system that may allow for future studies to confirm this newly identified role for the receptor. Although my original hypothesis regarding PXR involvement in hormonal control of the *UGT1* locus was proved invalid, the creation of a Tg-*UGT1*-PXRnull model

system for those experiments revealed new information about PXR and its central importance in UGT1A expression. The studies described in this dissertation provide the first evidence for a suppressive and tissue-specific function of PXR in control of human drug metabolism genes. However, further characterization of the molecular interactions between PXR and its coactivators or corepressors and PXR and its DNA response elements must be performed to verify that the results observed in this study are due to such interactions.

Despite the serendipitous discovery of a previously undescribed regulatory phenomenon within the data disproving my original hypothesis, the question of how hormonal changes translate to the dynamic UGT1A upregulation observed during pregnancy and estrus remains. In the last two years, additional work was performed in the laboratory regarding sexually dimorphic expression of the UGT1As. Differential expression between male and female mice and rats has been well-characterized for the phase I cytochromes P450, and results from the temporal differences in pulsatile secretion of growth hormone during development. The growth hormone receptor activates the small transcription factor STAT5, which in turn activates male- or female-specific transcription factors that can “turn on” or “turn off” transcription of specific CYPs. A recently published study revealed a similar effect of growth hormone on gender-dimorphic mouse *Ugt* expression (Buckley and Klaassen, 2009). The *UGT1A* promoter regions are riddled with STAT5 consensus sites as determined by sequence homology, and these data together suggest that the male/female differences observed in UGT1A expression are likely due to the same STAT5 pathway demonstrated to mediate dimorphic CYP expression. Growth

hormone is not the only nuclear hormone receptor capable of activating the STAT5 pathway, however. Prolactin and placental lactogens, acting through the prolactin receptor (PLR), have been shown not only to activate STAT5 but also to induce transcription of the phase III bile salt transporter NTCP (Cao J et al., 2001). PR is also expressed in rodent liver and is highly induced during pregnancy. Therefore, this is an exceptional candidate pathway for UGT1A upregulation during pregnancy and lactation, and also correlates with a Proestrus prolactin surge that may account for the increased transcriptional activity that begins in Proestrus and peaks during Estrus and Diestrus I. The availability of STAT5a/b knockout mice would enable conclusive in vivo evidence of the contribution of this pathway toward the hormonal regulation observed in the Tg-*UGT1* mice.

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