ABSTRACT

JACKSON, JONATHAN PATRICK. The Role of the Nuclear Receptors CAR and PXR in the Drug Induced Transcriptional Regulation of the Murine CYP2C Subfamily of Cytochromes P450 Monooxygenases. (Under the direction of Joyce A. Goldstein and Randy L. Rose.)

The CYP2C subfamily of cytochrome P450 monoxygenases (P450) is responsible for the metabolism of approximately 20% of therapeutic drugs including the anticonvulsant phenytoin. Recently, phenytoin has been reported to induce the expression of the human genes *CYP2B6*, *CYP3A4*, and *CYP2C8* and the murine gene *Cyp2c29*. Cytochrome P450 expression is often induced by prior exposure to xenobiotics resulting in drug-drug interactions and changes in drug efficacy. Induction of several human CYP2C enzymes by xenobiotics occurs at the transcriptional level and is reported to involve the constitutive androstane receptor (CAR) and/or the pregnane X receptor (PXR). However, the molecular mechanisms regulating drug induction of the murine CYP2C enzymes remain unclear. The mouse is an excellent model system to investigate CYP2C drug induced transcription due to the availability of nuclear receptor knockout mice. Herein, we report the identification of two phenobarbital and phenytoin inducible murine CYP2C genes, *Cyp2c29* and *Cyp2c37*. Quantitative RT-PCR demonstrates that hepatic CYP2C29 and CYP2C37 mRNA is induced by phenobarbital and phenytoin. Additionally, immunoblots indicated that phenytoin induced hepatic CYP2C29 and CYP2C37 protein. We utilized *in vivo* and *in vitro* gene reporter assays of the *Cyp2c29* promoter to delineate the phenytoin-response activity to a phenytoin responsive module (PHREM) located -1371 bp upstream of the *Cyp2c29* translation

start site. Similarly, using *in vitro* gene reporter assays of the *Cyp2c37* promoter, we identified a single functional CAR-RE located -2791 bp upstream of translation start site of *Cyp2c37*. Mutagenesis studies demonstrated that these sites are essential for CAR transactivation of their respective gene promoters in HepG2 cells. Using quantitative RT-PCR, we demonstrated that induction of CYP2B10 mRNA by phenytoin was completely abolished in CAR-null mice, but only moderately reduced in PXR-null mice. Similarly, phenytoin induction of CYP2C29 and CYP2C37 mRNA was almost completely removed in CAR-null mice and only modestly reduced in PXR-null mice. Taken together, these results indicate that the induction of Cyp2b10, Cyp2c29, and Cyp2c37 by phenytoin is predominately regulated by mCAR. However, induction of CYP3A11 mRNA was only partially decreased in either CAR-null or PXR-null mice. Studies have demonstrated that CYP3A11 mRNA is induced by CAR and PXR agonists, thus our results also indicate that phenytoin acts as an agonist of both CAR and PXR in a promoter dependent manner.

THE ROLE OF THE NUCLEAR RECEPTORS CAR AND PXR IN THE DRUG INDUCED TRANSCRIPTIONAL REGULATION OF THE MURINE CYP2C SUBFAMILY OF CYTOCHROMES P450 MONOOXYGENASES

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A dissertation submitted to the Graduate
Faculty of North Carolina State University
in partial fulfillment of the requirements
for the Degree of Doctor of Philosophy

TOXICOLOGY

Raleigh

2006

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I dedicate this work to my mother and father.

Who prepared me to achieve excellence in life by understanding that education is of primary importance and that integrity, determination, and patience are all qualities that one needs to excel in life.

BIOGRAPHY

I am Jonathan Patrick Jackson, the son of Jeanette Glenn and Rickman P. Jackson. I was born and raised in the beautiful southern Appalachian Mountains of western North Carolina in the city of Hendersonville. Hendersonville is a small tourism and agricultural community well known for mountain golf courses, the Carl Sandburg home site, the Flat Rock Play House, and the annually held Apple Festival.

Living in the rural mountains of North Carolina provided me with the opportunity to develop a love for several outdoor hobbies that I spent a large amount of my free time enjoying while growing up. I enjoyed backpacking, horseback riding, rock climbing, golf, fishing, and hunting. I enjoyed all of these hobbies immensely, but preferred horseback riding because it was a family affair. My parents were both from large, tight knit families and felt strongly about spending quality time with family. Both my parents were from blue-collared families that worked in the local industrial mills located in and around Hendersonville. The salaries of my parents provided a modest, but adequate lifestyle for our family of four. I was blessed with one older sibling, Vicki D. Jackson Kilpatrick. My sister and I had to overcome a considerable age difference of ~6 years during our childhood that at times made both our lives challenging. However, we are now very close and have come to understand that time changes everything. During my adolescence, my parents were excellent role models and continually reinforced the ideology that good things come to those that work the hardest. This ideology suggests that a person that is persistent, patient,

and of high integrity can excel and achieve many goals in life. I quickly became to understand these principles and readily applied them to my studies.

I spent the first 18 years of my life in Hendersonville, where I graduated from East Henderson High School. I then traveled to Raleigh, North Carolina on a scholarship to attend North Carolina State University in the Fall of 1995. I completed my Bachelor of Science in Animal Science and intended to apply to the school of veterinarian medicine. However during my undergraduate degree program, I became less interested in veterinarian medicine and more interested in basic scientific research.

During my last semester at North Carolina State University, I was fortunate to cross paths with Dr. Ronald Cannon. Dr. Cannon, a guest lecturer from the National Institute of Environmental Health Sciences (NIEHS), was presenting a lecture in a genetics course that I was enrolled in and informed our class that he had a job opportunity in his laboratory. I inquired about the job opportunity, interviewed and was hired into a one year temporary training position. His lab was investigating the molecular mechanisms regulating squamous cell papilloma formation following chemical promotion within the TgAc transgenic mouse strain. I was given a small independent project that coincided with the main focus of the lab. This position provided me with a useful array of molecular biology techniques and inspired me to apply to graduate school.

I applied to the Department of Environmental and Molecular Toxicology at North Carolina State University to obtain a Masters in Toxicology. Funding was difficult to obtain, but luckily I was given the opportunity to become a teaching

assistant for the Department of Biological Sciences to support myself. Teaching was a rewarding experience and I gained a new perspective from observing the classroom setting from the other side of the aisle. Not long into my graduate studies, I encountered Dr. Joyce A. Goldstein presenting a lecture on Pharmacogenetics. I was immediately interested in her work because of the severe reactions associated with drug therapy in some patients as a result of defective cytochromes P450 monooxygenases enzymes. I approached her to inquire about a summer internship in her laboratory at the NIEHS and was rewarded with a 3 month temporary position in her laboratory studying single nucleotide polymorphisms found in the coding sequence of the *CYP2C19* gene. During my internship, I was given the opportunity to significantly contribute to the project and received a co-authorship on the subsequent publication. Upon completion of my internship, Dr. Goldstein spoke with me at length, inquiring about my future plans and goals. During our conversation, Dr. Goldstein offered to fund my dissertation research to obtain my Doctorate of Philosophy in Toxicology. I happily accepted and have enjoyed great success under the tutelage of Dr. Goldstein and her current staff.

During my long educational career, I have used the principles that my parents instilled in me of patience and determination to achieve my goals. However, I have become to realize in life that even the best laid plans can be disrupted by the unpredictable nature of life. No one can achieve success without remaining patient and determined, but one must also have some strokes of good fortune. I am thankful to have been so fortunate in my endeavors.

ACKNOWLEDGEMENTS

I am indebted to Dr. Joyce A. Goldstein for having confidence in my potential and for providing me the opportunity to pursue my doctorate in her laboratory. I would also like to thank Dr. Goldstein for granting me a measured amount of independence in the undertaking of my dissertation research as this has helped me to develop great self-confidence in my ability as a scientist. I have thoroughly enjoyed my experience in her laboratory and will leave adequately prepared to become successful in the next stage of my scientific career. I would like to acknowledge Dr. Goldstein's current staff; Sherry Coulter, Su-jun Lee, Yu-ping Chen, Joyce Blaisdell, and Tracy Delozier, for their support, instruction, and counsel during my tenure. I would like to especially thank Dr. Stephen S. Ferguson for his teaching prowess in experimental design and data analysis which was instrumental in the commencement and completion of my dissertation research.

I would also like to acknowledge several individuals that have provided instruction to me in the performance of several molecular biological techniques during my research. I would like to thank Kaoru Inoue and Sherry Coulter for their expert help in Western blot analysis, Donna Mays for her knowledge in quantitative RT-PCR, Tatsu Seuyoshi for his knowledge in nuclear protein isolation and electrophoretic mobility shift assays, and Rick Moore for his instruction in tail vein injections. I would like to thank these individuals for their time, energy, and patience while I became efficient at their respective techniques.

In addition, I would like to acknowledge the animal facility staff for their professional manner and instruction on animal research procedures at the National Institute of Environmental Health Sciences. I would like to thank Louise Harris, animal facility technician, for animal drug administration and her immediate supervisor, Al Calviness, for coordinating animal housing and dosing regiments.

Finally, I would like to recognize my doctoral committee members: Dr. Randy L. Rose, Dr. Ernest Hodgson, Dr. Masahiko Negishi, and Dr. Andrew D. Wallace. I would like to thank the committee for their continued support and their contributions to my dissertation research.

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1.0 Introduction

All biological organisms are continuously exposed to foreign and endogenously synthesized toxicants. Foreign toxicants, also known as xenobiotics, include manufactured and natural chemicals such as drugs, industrial chemicals, pesticides, pollutants, and toxins produced by fungi, plants, and animals (Casarett and Doull, 2001 3-34; Hodgson and Smart, 2001 1-10; Bohan and Boyer, 2002; Liddle and Goodwin, 2002). Organisms are commonly exposed to xenobiotics via inhalation, ingestion, and dermal absorption. Most xenobiotics are lipid soluble, which facilitates their absorption across biological membranes. Xenobiotics with these properties have the potential to accumulate in target tissues in high concentrations, resulting in organ toxicity and/or death (Hodgson and Levi, 1997 27-57; Casarett and Doull, 2001 35-132; Hodgson and Smart, 2001 1-10). Organisms have developed two mechanisms to defend against the accumulation of toxic lipophilic compounds, namely biotransformation and transport (Hodgson and Levi, 1997 57-118; Casarett and Doull, 2001 107-224; Hodgson and Smart, 2001 67-135; Liddle and Goodwin, 2002; Pascussi et al., 2004). These two mechanisms work in a concerted effort to decrease systemic availability and residence time of toxicants.

The liver is the primary organ of uptake, biotransformation, and excretion of xenobiotics. This is due to its location and the expression of a large number of xenobiotic-metabolizing enzymes and transport proteins (Hodgson and Levi, 1997 57-118; Casarett and Doull, 2001 471-489; Hodgson and Smart, 2001 1-10; Bohan and Boyer, 2002). The liver is strategically positioned between the portal and

systemic blood circulations; thus xenobiotics absorbed through the gut are frequently altered before entering the systemic circulation (Hodgson and Levi, 1997 57-118; Casarett and Doull, 2001 107-132; Hodgson and Smart, 2001 67-135; Liddle and Goodwin, 2002). In hepatocytes, xenobiotics are enzymatically modified to increase their water solubility by a process known as biotransformation. As a consequence, the physiochemical properties of the xenobiotics are changed, favoring excretion over absorption, thus resulting in detoxification. However, it is important to recognize that chemical modification of a xenobiotic changes its biological effect thus a more or less toxic metabolite is possible. This fact is an important principle in Pharmacology and Toxicology. Some pharmaceutics must undergo biotransformation to exert their therapeutic effects. Similarly, many xenobiotics must be biotransformed to exert their characteristic toxic or carcinogenic effect (Hodgson and Levi, 1997 95-105; Casarett and Doull, 2001 133-224; Hodgson and Smart, 2001 1-10; Johnson and Stout, 2005). To decrease the impact of xenobiotic metabolites on cellular processes, transport proteins also facilitate their removal from the hepatocyte via the blood or bile using various transport mechanisms (Bohan and Boyer, 2002).

Both xenobiotic metabolism enzymes and transport proteins collaborate to produce an effective protective system against the toxic accumulation of xenobiotics. The cytochrome P450 monooxygenases (P450) play an integral role in this protective system. P450s are heme-containing enzymes that are extensively expressed in hepatocytes. Hepatic phase I xenobiotic metabolism is primarily performed by P450 enzymes, which execute several types of oxidation reactions

such as aliphatic hydroxylation, ester cleavage, and oxidative group transfer (Casarett and Doull, 2001 133-224; Hodgson and Smart, 2001 67-113). P450 enzymes are extremely broad in their substrate specificity and so metabolize many structurally diverse endogenous and exogenous substrates. Currently, there are 17 different P450 gene families encoded by approximately 60 genes in any given mammalian species (Nelson, 1999; Waxman, 1999; Guengerich et al., 2005; Johnson and Stout, 2005).

Xenobiotic metabolism in hepatocytes is often affected by the expression of P450 enzymes. It is known that prior treatment with certain foreign chemicals can induce many of the P450 genes included in the CYP1-4 gene families (Honkakoski et al., 1998a; Goodwin et al., 1999; Sueyoshi et al., 1999; Goodwin et al., 2001; Raucy et al., 2002; Raucy, 2003; Pascussi et al., 2004). The CYP1A genes have been shown to be induced by the polycyclic aromatic hydrocarbons (PAH), TCDD, and some polychlorinated biphenyls (PCB) (Poland and Glover, 1974; Nebert and Jones, 1989; Neuhold et al., 1989; Okey, 1990). The fibrate class of drugs have been shown to induce the CYP4A genes (Sharma et al., 1988; Johnson et al., 1996; Waxman, 1999). Phenobarbital (PB), phenytoin, and rifampicin have all been shown to induce many of the CYP2B, CYP3A, and CYP2C genes (Honkakoski et al., 1998a; Honkakoski et al., 1998b; Goodwin et al., 1999; Sueyoshi et al., 1999; Gerbal-Chaloin et al., 2001; Goodwin et al., 2001; Goodwin et al., 2002; Raucy et al., 2002; Raucy, 2003; Wang et al., 2003a; Chen et al., 2004; Ferguson et al., 2005). The induction of P450 enzymes can be beneficial by increasing the rate of the detoxification process. However, induction of P450 enzymes is also associated

with both pharmacological and toxicological consequences such as alterations in drug efficacy, drug-drug interactions, and metabolic activation of procarcinogens (Thummel and Wilkinson, 1998; Waxman, 1999; Sueyoshi and Negishi, 2001). The inducible CYP1-4 P450 gene families code for enzymes that predominately metabolize therapeutics and environmental chemicals (Waxman, 1999; Guengerich et al., 2005).

1.1 Drug Induction Mechanisms of the P450 Genes

Drug induction of P450 genes is frequently regulated at the transcriptional level and is mediated by receptor-dependent mechanisms (Waxman, 1999; Honkakoski and Negishi, 2000; Sueyoshi and Negishi, 2001; Pascussi et al., 2004). These receptors are ligand activated DNA-binding transcription factors that activate a battery of genes in response to external or intracellular stimuli. The activity of these receptors is governed by the binding of small, generally lipophilic ligands including hormones, fatty acid derivatives, bile salts, oxysterols, endobiotics, and xenobiotics (Waxman, 1999; Honkakoski and Negishi, 2000; Sueyoshi and Negishi, 2001; Pascussi et al., 2004). Once activated, many receptors dimerize with an obligate protein partner before binding their cognate DNA response element within the 5'-flanking region of target genes. Subsequently, these proteins recruit co-activators to facilitate the initiation of transcription (Waxman, 1999; Honkakoski and Negishi, 2000; Sueyoshi and Negishi, 2001; Pascussi et al., 2004). The activities of co-activators include chromatin remodeling, recruitment of other cofactors, and recruitment of the basal transcription protein complex (Honkakoski and Negishi,

2000; Pascussi et al., 2004). The interaction of these coactivators with the activated receptors and the basal transcription machinery contribute to the full activation of gene transcription and expression. Transcriptional activation of P450 genes by xenobiotics is predominately governed by two main groups of receptors: the aryl hydrocarbon receptor (AhR), a member of the Per/Arnt/Sim (PAS) family of transcription factors and the xenobiotic sensing nuclear receptors (Rowlands and Gustafsson, 1997), the constitutive active/androstane receptor (CAR, NR1I3) and the pregnane X receptor (PXR, NR1I2) (Honkakoski and Negishi, 2000; Sueyoshi and Negishi, 2001; Liddle and Goodwin, 2002).

The AhR is a member of the basic helix-loop helix PAS family of transcription factors, which include hypoxia inducible factors 1-3 (Hif1-3), genes involved in the circadian rhythm Per and Clock, and some co-activators of transcription such as SRC-1 and TIF2 (Rowlands and Gustafsson, 1997; Waxman, 1999; Pascussi et al., 2004). The AhR becomes activated in the cytosol by binding PAH and TCDD. The activated receptor translocates into the nucleus and heterodimerizes with AhR nuclear translocator protein (Arnt) forming a functional transcription factor complex (Fig. 1.1) (Poland et al., 1976; Okey et al., 1979; Nebert and Jones, 1989; Neuhold et al., 1989; Okey, 1990; Hoffman et al., 1991; Reyes et al., 1992; Hankinson, 1995). The protein complex binds to xenobiotic response elements (XRE), a conical sequence described as “5’-CACGCNA/T-3’”, and then recruits several co-activators to stimulate the transcription of target genes. AhR controls the induction of the CYP1A P450 genes by PAH, TCDD, and some PCB (Hankinson, 1995; Rowlands and Gustafsson, 1997).

The xenobiotic sensing nuclear receptors CAR and PXR belong to a superfamily of nuclear receptors which include the vitamin D receptor (VDR, NR1I1), thyroid receptor (NR2C1-2), peroxisome proliferator-activated receptor (PPAR, NR1C1-3), and the glucocorticoid receptor (GR, NR3C1). Currently, there have been over 48 nuclear receptors identified in humans (Mangelsdorf et al., 1995; Committee, 1999; Chawla et al., 2001; Makishima, 2005). All nuclear receptors share a similar modular structure composed of four domains which are involved in the function of these receptors. These domains are the N-terminus AF-1 ligand-independent transactivation domain, the DNA-binding domain (DBD) that consists of two highly conserved zinc finger motifs, the ligand-binding domain (LBD), and the C-terminus ligand-dependent AF-2 transactivation domain (Fig. 1.2) (Mangelsdorf et al., 1995; Honkakoski and Negishi, 2000; Chawla et al., 2001; Pascussi et al., 2004). The activity of these nuclear receptors is modulated by ligands such as fatty acids, bile acids, steroids, xenobiotics, and other lipid soluble compounds. Once activated by bound ligand, several of these nuclear receptors form heterodimers with the retinoid X receptor (RXR) or homodimers before binding their cognate DNA response elements (Mangelsdorf et al., 1995; Honkakoski and Negishi, 2000; Chawla et al., 2001; Pascussi et al., 2004). Ligand binding also induces a conformational change which repositions the AF-2 domain allowing the nuclear receptor to interact with co-activators such as SCR-1, TIF2, and GRIP (p160 family) (Mangelsdorf et al., 1995; Honkakoski and Negishi, 2000; Chawla et al., 2001; Pascussi et al., 2004; Makishima, 2005). Some co-activators possess intrinsic histone acetyltransferase activity (HAT) which relieves the suppressive chromatic

effects and activates transcription. However, some nuclear receptors without bound ligand negatively regulate gene transcription by recruiting co-repressors such as N-CoR and SMRT (Honkakoski and Negishi, 2000; Pascussi et al., 2004). These co-repressors recruit histone deacetylases to induce chromatin condensation which represses transcription (Pascussi et al., 2004). All nuclear receptors act as bioactive molecular sensors to elicit changes in gene expression. Most nuclear receptors are activated by only one class of bioactive molecules such as the activation of GR by glucocorticoids. However, the nuclear receptors CAR and PXR are unique in their ability to respond to a diverse range of chemical compounds.

1.2 The Nuclear Receptor CAR

The nuclear receptor CAR was first characterized as a transactivator of retinoid acid response elements (RARE) (Baes et al., 1994). The term "constitutive" activator was coined due to the receptor's ability to activate RARE in the absence of ligand. It was later named the "constitutive androstane receptor" when it was discovered that CAR bound two stereospecific androstane steroid derivatives, androstanol (5α-androstan-3α-ol) and androstenol (5α-androst-16-en-3α-ol) (Forman et al., 1998). Within the same year, CAR was identified as the key regulator of PB induction of the murine and human CYP2B genes by Negishi and co-workers (Honkakoski et al., 1998b; Sueyoshi et al., 1999). They demonstrated that CAR in response to PB bound as a heterodimer with RXR to the phenobarbital responsive enhancer module (PBREM) within the 5'-flanking region of the murine *Cyp2b10* and human *CYP2B6* genes. These studies were later supported by data from the

recently derived transgenic CAR-null mice. PB induction of the *Cyp2b10* gene was abolished in these mice, clearly demonstrating that CAR mediates PB induction of the Cyp2B genes (Wei et al., 2000) Recently, CAR has been shown to be involved in the transcriptional regulation of other CYP enzyme subfamilies, including CYP3A and the CYP2C (Ferguson et al., 2002; Gerbal-Chaloin et al., 2002; Goodwin et al., 2002; Chen et al., 2003; Ferguson et al., 2005).

CAR is primarily expressed in liver and intestine (Baes et al., 1994; Pascussi et al., 2004). In untreated, normal hepatocytes CAR is sequestered in the cytosol (Kawamoto et al., 1999; Honkakoski and Negishi, 2000). In response to activators such as PB CAR translocates to the nucleus (Fig. 1.3), forms a functional transcriptionally active heterodimer with RXR, and binds hexameric direct repeats of the consensus sequence "AGGTCA" separated by 4 nucleotides (DR4) found in the 5'-flanking regions of such CAR-responsive genes as *Cyp2b10* and *CYP2B6* (Kawamoto et al., 1999; Honkakoski and Negishi, 2000; Sueyoshi and Negishi, 2001; Pascussi et al., 2003; Pascussi et al., 2004). CAR has also been shown to bind nuclear receptor response elements organized as DR-3 motifs or everted repeats of the consensus sequence "AGGTCA" spaced by 6 nucleotides (ER6) such as in the *CYP3A4* gene (Goodwin et al., 2002; Frank et al., 2003).

The molecular mechanism regulating the retention of CAR in untreated hepatocytes and subsequent nuclear translocation of activated CAR is not entirely understood, but phosphorylation is believed to be involved. Treatment with okadaic acid (OA), a protein phosphatase inhibitor, prevents PB induction of the *Cyp2b10* gene in murine hepatocytes (Shimada et al., 1994; Honkakoski and Negishi, 1998;

Honkakoski and Negishi, 2000). In murine primary hepatocytes, OA treatment also represses PB induced nuclear translocation of CAR (Kawamoto et al., 1999; Honkakoski and Negishi, 2000). However in HepG2 cells, the cytosolic retention mechanism is lost and as a result transiently transfected CAR translocates immediately to the nucleus (Honkakoski and Negishi, 2000). Recently, Negishi and co-workers demonstrated that in non-induced murine hepatocytes CAR is in a tubulin associated ternary protein complex with heatshock protein 90 (hsp90) and the newly discovered tetratricopeptide repeat (TPR) protein, designated cytoplasmic CAR retention protein (CCRP) (Kobayashi et al., 2003; Yoshinari et al., 2003). They further demonstrated in primary murine hepatocytes that PB treatment elicits the recruitment of protein phosphotase 2A (PP2A) to the CAR cytoplasmic protein complex (Fig. 1.3) (Yoshinari et al., 2003). Although this is consistent with OA inhibition of CAR nuclear translocation, it is still unknown whether the recruitment of PP2A is necessary for CAR nuclear translocation. Furthermore, the over-expression of CCRP represses the drug elicited nuclear translocation of CAR (Kobayashi et al., 2003). These questions suggest that other factors are involved in this poorly understood mechanism governing cellular compartmentalization of CAR.

CAR is highly constitutively active in the absence of ligand. This is a unique characteristic among nuclear receptors. The activity of most nuclear receptors is ligand-dependent. However, the ligand-dependent coactivator binding surface or AF-2 domain within CAR is not dependent on the binding of agonist ligands to appropriately position the C-terminal LXXLL motif into an active conformation (Pascussi et al., 2004; Moore, 2005). In the active conformation the LXXLL motif of

the AF-2 domain promotes interaction of co-activators via their conserved LXXLL motifs (Pascussi et al., 2004; Moore, 2005). The active conformation of CAR is promoted by two unique features within its AF-2 domain, 1) the flexible loop that connects Helix 10 to the C-terminal LXXLL motif in other nuclear receptors is replaced by a short helix that holds the AF-2 domain C-terminus in place more rigidly (Moore, 2005) and 2) the AF-2 domain C-terminus of CAR is missing an amino acid sequence extension which allows the terminal carboxyl group of CAR to form hydrogen bonds with adjacent helices. These interactions hold CAR in the active conformation by fastening the AF-2 domain to the LBD (Fig. 1.4) (Dussault et al., 2002; Moore, 2005).

The binding of androstenol undermines this mechanism, causing the constitutive transcriptional activity of CAR to be inhibited. Androstenol and androstanol are examples of naturally occurring inverse agonists that exert their inhibitory effects by promoting the release of the co-activator SRC-1 from the AF-2 domain of CAR (Forman et al., 1998; Pascussi et al., 2004; Moore, 2005). Bound androstenol destablizes the hydrogen bonding holding the AF-2 domain of CAR in the active conformation (Shan et al., 2004; Moore, 2005). The change in conformation allows the interactions of co-repressors, thus decreasing the activity of CAR (Shan et al., 2004; Moore, 2005).

The inhibitory effects of the androstanes on the constitutive activity of mouse CAR (mCAR) can be reversed by the binding of the potent PB-like mCAR agonist 1, 4-bis-[2-(3, 5,-dichloropyridyloxy)] benzene (TCPOBOP) (Sueyoshi et al., 1999; Tzameli et al., 2000; Sueyoshi and Negishi, 2001; Moore, 2005). The binding of

TCPOBOP induces the recruitment of co-activators such as SRC-1. Interestingly, TCPOBOP has been shown to superactivate mCAR in transfection assays (Sueyoshi et al., 1999; Tzameli et al., 2000; Sueyoshi and Negishi, 2001; Moore, 2005). It is thought that TCPOBOP makes direct contacts with the short linker helix and helix 12 within the AF-2 domain of CAR. These interactions further stabilize the AF-2 domain in the active conformation which favors the recruitment of co-activators (Suino et al., 2004; Moore, 2005).

The nuclear accumulation of CAR has been shown to be induced by several compounds such as PB, bilirubin, and bile acids (CAR activators) which do not bind CAR (Swales and Negishi, 2004). The activation of CAR target genes in response to CAR activators is clearly dependent upon the constitutive activity of CAR. The direct ligand TCPOBOP initiates nuclear translocation and acts as a conventional agonist that has been shown to superactivate CAR, thus it is not solely dependent upon the constitutive activity of CAR to activate gene expression. The differences in the effects of CAR activators and direct ligands such as TCPOBOP on the behavior of mCAR have indicated that the activation of CAR and its genes is governed by two mechanisms: nuclear translocation and ligand binding.

1.3 The Nuclear Receptor PXR

PXR is closely related to CAR, sharing ~70% and ~50% sequence identity to the DBD and LBD of CAR (Fig 1.5), respectively (Kliewer et al., 2002; Moore et al., 2003). PXR was first identified by Kliewer et al. (1998) as a novel orphan nuclear receptor activated by the pregnane steroids. The identification of PXR was based its

sequence homology with other nuclear receptors. During the same year, Bertilsson et al. (1998) and Blumberg et al. (1998) isolated and characterized the same receptor but designated the receptor as SXR (steroid and xenobiotic receptor) and PAR (pregnane activated receptor), respectively. These groups mainly observed the expression of PXR in the liver and intestine (Bertilsson et al., 1998; Blumberg et al., 1998; Kliewer et al., 1998; Pascussi et al., 2003; Pascussi et al., 2004). Together, these studies were the first to identify PXR as the nuclear receptor mediating CYP3A drug induction. The introduction of the transgenic PXR-null mice provided additional evidence to support these early studies, clearly demonstrating that PXR mediated CYP3A drug induction (Staudinger et al., 2001a; Staudinger et al., 2001b; Kliewer et al., 2002).

PXR has been identified in several species other than mouse such as human, rat, dog, rabbit, and *xenopus laevis* (LeCluyse, 2001; Kliewer et al., 2002; Moore et al., 2003; Pascussi et al., 2003; Pascussi et al., 2004). The identification of PXR orthologs in multiple species indicates that the signaling pathway mediated by this receptor was conserved during species evolution. Interestingly, there are distinct species differences in the induction of CYP3A genes by xenobiotics. These differences were later correlated to differences in the xenobiotic activation profiles of the varying PXR orthologs among species (LeCluyse, 2001; Kliewer et al., 2002). For example, the expression of the human CYP3A4 and the rabbit CYP3A6 is strongly induced by rifampicin, an efficacious activator of human and rabbit PXR. In the mouse, however, pregnenolone-16α-carbonitrile (PCN) and not rifampicin strongly induces the expression of CYP3A11 (LeCluyse, 2001; Kliewer et al., 2002).

An alignment of PXR LBD sequences indicate that these sequences have diverged through the evolution of species, thus it is reasonable to observe species differences in the activation profiles of PXR (Fig. 1.5) (LeCluyse, 2001; Kliewer et al., 2002; Moore et al., 2002).

Unlike CAR, PXR is a constitutive inactive receptor that requires ligand binding to become activated. PXR binds a wide variety of structurally diverse endogenous or exogenous compounds including drugs such as rifampicin, phenobarbital, steroid hormones and derivatives, and dietary compounds such as hyperforin (Bertilsson et al., 1998; Blumberg et al., 1998; Kliewer et al., 1998; Lehmann et al., 1998; Pascussi et al., 2003; Pascussi et al., 2004). Analysis of the crystal structure of PXR has revealed key factors within the LBD that are responsible for its promiscuity. The presence of a flexible loop within the LBD allows PXR to bind ligands of varying size and in multiple orientations, since the loop may expand or contract (Watkins et al., 2001; Kliewer et al., 2002; Pascussi et al., 2003; Pascussi et al., 2004; Kretschmer and Baldwin, 2005). Also, the LBD of PXR contains fewer polar groups which allow the receptor to interact with a larger range of compounds (Watkins et al., 2001; Kliewer et al., 2002; Pascussi et al., 2003; Pascussi et al., 2004; Kretschmer and Baldwin, 2005).

PXR has been shown to mediate the drug induction of several P450 genes including *CYP3A4*, *Cyp3a11*, *CYP2B6*, *CYP2C8*, *CYP2C19* and *CYP2C9* (Gerbal-Chaloin et al., 2001; Goodwin et al., 2001; Chen et al., 2003; Wang et al., 2003a; Wang et al., 2003b; Chen et al., 2004; Ferguson et al., 2005). Drugs such as rifampicin and hyperforin have been shown to induce *CYP3A4*, *CYP2B6*, *CYP2C8*,

CYP2C19 and *CYP2C9* genes (Gerbal-Chaloin et al., 2001; Goodwin et al., 2001; Chen et al., 2003; Wang et al., 2003a; Wang et al., 2003b; Chen et al., 2004; Ferguson et al., 2005). These drugs initiate a cascade of events that starts with the ligand-dependent activation of PXR. Once activated, PXR translocates to the nucleus forming a heterodimer with RXR and then associates with co-activators such as SRC-1 (Kliewer et al., 1998; Kliewer et al., 2002; Pascussi et al., 2003; Pascussi et al., 2004). Subsequently, this fully functional ligand-dependent transcription factor binds response elements located within the 5'-flanking regions of PXR-responsive genes (Fig. 1.6). PXR response elements consist of two AGGTCA hexanucleotide motifs organized in a DR-3 or as an ER-6, such as those found with the *CYP3A4* xenobiotic responsive enhancer module (XREM) (Kliewer et al., 1998; Goodwin et al., 1999; Kliewer et al., 2002). PXR has also been shown to interact with nuclear response elements organized as a DR-4 motif such as those found in the PBREM of *CYP2B6* (Goodwin et al., 2001; Kliewer et al., 2002; Wang et al., 2003a).

The nuclear translocation of PXR is a controversial topic; however, two independent studies have demonstrated that PXR is retained in the cytosol of untreated murine hepatocytes until treated with PCN. The treatment of murine hepatocytes with PCN resulted in the nuclear accumulation of PXR (Kawana et al., 2003; Squires et al., 2004). Squires et al (2004) showed that the nuclear accumulation of PXR was subsequently followed by an increase in murine CYP3A11 mRNA. This study established that nuclear translocation of PXR is a primary step in the activation of PXR-responsive genes.

1.4 The Functional Overlap Between CAR and PXR

Originally CAR and PXR were identified to mediate xenobiotic induction of the CYP2B and CYP3A genes, respectively. CAR was shown to regulate CYP2B induction by binding the PBREM within the 5'-flanking region of the CYP2B genes, *Cyp2b10* and *CYP2B6* (Honkakoski et al., 1998b; Sueyoshi et al., 1999; Honkakoski and Negishi, 2000; Sueyoshi and Negishi, 2001). Similarly, PXR was demonstrated to bind the XREM within the 5'-flanking sequence of the *CYP3A4* gene resulting in increased transcription (Goodwin et al., 1999). These pathways appeared to be distinctly different pathways based upon the clear differences in the architecture of the PBREM and XREM nuclear receptor binding motifs. The PBREM found in the *Cyp2b10* and *CYP2B6* genes is composed of two DR-4 motifs, whereas the XREM of the *CYP3A4* gene consists of a DR-3 motif and an ER-6 motif (Honkakoski et al., 1998a; Honkakoski et al., 1998b; Goodwin et al., 1999; Goodwin et al., 2002). Recently, PXR and CAR have been shown to bind response elements in several different configurations such as DR-3, DR-4, DR-5, ER-6, and ER-8, which indicates that the DNA binding preferences of CAR and PXR are quite similar (Goodwin et al., 2001; Goodwin et al., 2002; Kliewer et al., 2002; Makinen et al., 2002; Frank et al., 2003). Similar studies indicate that the overlap of CAR and PXR target genes reaches beyond the CYP2B and the CYP3A genes, but also includes the CYP2C genes (Ferguson et al., 2002; Gerbal-Chaloin et al., 2002; Chen et al., 2003; Chen et al., 2004; Ferguson et al., 2005).

CAR and PXR have also been shown to share some activators such as PB, clotrimazole, and dieldrin with the caveat that species differences are observed (Fig

1.7) (Moore et al., 2000; Wei et al., 2002; Pascussi et al., 2003; Pascussi et al., 2004; Zhang et al., 2004). For example, the antifungal agent clotrimazole has been shown to induce the mouse gene *Cyp3a11* in both PXR-null and CAR-null mice, but this induction is abolished in the CAR/PXR double null mouse (Zhang et al., 2004). These results indicate that the induction of the *Cyp3a11* gene by clotrimazole is mediated by CAR and PXR. However, clotrimazole has been shown to antagonize hCAR, indicating that species differences do occur (Moore et al., 2000; Zhang et al., 2004).

The frequent cross-interaction of these distinct signaling pathways is the result of two distinct mechanisms: (1) CAR and PXR bind the same cis-acting elements, and (2) CAR and PXR share some ligands/activators (Moore et al., 2000; Pascussi et al., 2003; Zhang et al., 2004). The significant overlap in the genes that CAR and PXR regulate and the sharing of activators suggests that these distinctly different signaling pathways are redundant allowing each receptor to compensate for the loss of the other.

1.5 The Phenytoin Controversy

The anticonvulsant activity of sodium diphenyl hydantoinate or phenytoin was discovered by Merritt and Putman in 1938 (Van Allen, 1984). Their studies demonstrated that phenytoin was a highly effective anticonvulsant that lacked the sedative properties of PB. These properties made phenytoin a mainstay therapeutic for over 52 years in the treatment of convulsive disorders (Van Allen, 1984). However, over the last decade several new antiepileptic drugs (AED) have been

introduced in an effort to reduce potential drug-drug interactions that are often associated with the older AEDs such as phenytoin (Asconape, 2002; Hachad et al., 2002). These drug-drug interactions frequently are the result of inhibition or induction of drug metabolizing enzymes. Although newer AED have been introduced, phenytoin continues to be commonly used (Asconape, 2002; Hachad et al., 2002).

Phenytoin is a known substrate of CYP2C9 and CYP2C19 (Goldstein and de Morais, 1994; Goldstein and Blaisdell, 1996; Miners and Birkett, 1998). The CYP2C subfamily plays a significant role in the metabolism of currently marketed drugs such as the anticoagulate warfarin, the diuretic torsemide, and several nonsteroidal anti-inflammatory drugs such as diclofenac, ibuprofen, and acetylsalicylic acid (Goldstein and de Morais, 1994; Goldstein and Blaisdell, 1996; Miners and Birkett, 1998). Current studies have demonstrated that the CYP2C subfamily of P450 enzymes accounts for ~20% of the total P450 content in human liver and is thought to metabolize ~20% of known drugs (Shimada et al., 1994; Goldstein, 2001). Frequently substrates are inducers of the enzymes that mediate their metabolism. Although phenytoin has not been shown to induce the expression of CYP2C9 and CYP2C19, it has been recently shown to induce CYP2C8 mRNA in primary human hepatocytes in our laboratory (Ferguson et al., 2005). These genes have been shown to share similar drug induction mechanisms, thus phenytoin may induce *CYP2C9* and *CYP2C19*. In a similar study, immunoblot analysis of mouse hepatic microsomes suggested that the major murine CYP2C protein, CYP2C29, was induced by phenytoin (Meyer et al., 2001a; Meyer et al., 2001b).

In other studies, phenytoin was found to induce the CYP3A and CYP2B genes in hepatocytes (Luo et al., 2002; Raucy, 2003; Wang et al., 2003b). Raucy et al. (2003) showed that phenytoin produced a marked increase in CYP3A4 mRNA, equivalent to that seen with rifampicin. Wang et al. (2003b) showed that CYP2B6 protein was equally induced by phenytoin and rifampicin in primary human hepatocytes. Using a *CYP3A4* XREM luciferase reporter cotransfected with human PXR in HepG2 cells, reporter activation by phenytoin was surprisingly much weaker than that produced by rifampicin (Raucy, 2003). Similar results were obtained by Wang et al. (2003b), using a *CYP2B6* PBREM luciferase reporter cotransfected with human PXR in Huh7 cells. The results of both studies were consistent and seem to suggest that phenytoin induction of the CYP3A and CYP2B genes may be mediated in a PXR-independent manner. However, these studies lacked conclusive evidence to determine whether phenytoin induction of P450 genes is mediated by CAR or PXR.

1.6 The Murine CYP2C Genes

The human CYP2C genes (*CYP2C8*, *CYP2C9*, *CYP2C18*, *CYP2C19*) have been studied extensively. However, relatively little is known of their mouse counterparts in both drug metabolism and drug induction. Originally only five murine CYP2C genes were identified (*Cyp2c29*, *Cyp2c37*, *Cyp2c38*, *Cyp2c39*, *Cyp2c40*). These genes were cloned from a liver cDNA library and were found to be primarily expressed in the liver and intestine using conventional RT-PCR (Luo et al., 1998). In a similar study, these genes were demonstrated to be widely expressed in

extrahepatic tissues such as lung, kidney and heart (Tsao et al., 2001). However, the tissue distribution of each CYP2C gene was found to be unique. Recently, 10 additional murine CYP2C genes and 4 pseudogenes were identified by Wang et al. (Wang et al., 2004). This study also discovered that the murine CYP2C genes were organized into a gene cluster located on chromosome 19 (Fig. 1.8).

The murine CYP2C enzymes CYP2C29, CYP2C37, CYP2C38, CYP2C39, CYP2C40, CYP2C44, CYP2C50, CYP2C54, and CYP2C55 have all been shown to metabolize arachidonic acid to epoxyeicosatrienoic acids or hydroxyeicosatetraenoic acids (Luo et al., 1998; Tsao et al., 2001; DeLozier et al., 2004; Wang et al., 2004). These arachidonic acid metabolites are potent bioactive molecules that have been shown to regulate vascular tone, affect ion transport in the kidney, and possess anti-inflammatory properties (Zou et al., 1996; Campbell and Harder, 1999; Node et al., 1999). However, very little has been published on the induciblility of the murine CYP2C enzymes and their involvement in drug metabolism.

Our laboratory has examined tolbutamide metabolism by the murine CYP2C enzymes including CYP2C29, CYP2C37-40, CYP2C44, CYP2C50, CYP2C54, CYP2C55, CYP2C65, CYP2C66, and CYP2C70 using partially purified recombinant proteins. We demonstrated that tolbutamide was metabolized by CYP2C29 > CYP2C38 > CYP2C39 (unpublished data). The human CYP2C9 enzyme was shown to be 70-83% more effective (4.39 nmol/min/nmol) at metabolizing tolbutamide than these murine CYP2C isoforms. The metabolism of tolbutamide was not appreciable by any of the other CYP2C isoforms examined. A study by Meyer et al. (2001a) suggested that CYP2C29 mediates the 6-O-demethylation of

the coumarin derivative scoparone and that the metabolite was increased by phenytoin and phenobarbital. In addition, an immunoblot suggested that PB and phenytoin induced CYP2C29 protein expression in liver microsomes.

Taken together, these studies both indicated that murine CYP2C enzymes are capable of drug metabolism. Furthermore, the study by Meyer et al. (2001a) showed that CYP2C mediated drug metabolism is affected by prior drug treatment. These studies suggest that the murine CYP2C enzymes are inducible and function as drug metabolizing enzymes, thus are similar to their human counterparts and provide an excellent model system for the investigation of the CYP2C P450 gene subfamily.

1.7 Specific Aims

The introduction of phenytoin in 1938 revolutionized the treatment of convulsive disorders. Phenytoin was demonstrated to be a highly effective anticonvulsant that had relatively little hypnotic effect (Van Allen, 1984). These properties made phenytoin a highly desirable antiepileptic drug for over a half-century. However, phenytoin treatment was associated with drug-drug interactions resulting from the induction of P450 enzymes (Asconape, 2002; Hachad et al., 2002). Although newer antiepileptic drugs have been introduced to reduce these risks, phenytoin remains in wide use. Recent studies have shown that phenytoin induces the CYP3A, CYP2B, and CYP2C genes. The CYP2C enzymes are thought to metabolize ~20% of known drugs including phenytoin (Shimada et al., 1994;

Goldstein, 2001). Frequently substrates are inducers of the enzymes that mediate their metabolism.

Drug induction of the human CYP2C genes have been shown to be mediated by both xenobiotic sensing nuclear receptors CAR and PXR (Ferguson et al., 2002; Gerbal-Chaloin et al., 2002; Chen et al., 2003; Chen et al., 2004; Ferguson et al., 2005). Conversely, no conclusive evidence has been shown to indicate whether phenytoin induction is mediated by CAR or PXR. Given the recent development of the transgenic CAR-null and PXR-null mice strains, the murine model provides an excellent system to examine the molecular mechanisms regulating phenytoin induction of the CYP2C genes. The recent studies by Meyer et al. (2001a; 2001b) in mice liver microsomes suggested that the expression of the CYP2C29 protein was induced by phenytoin and PB. However, this preliminary conclusion was based on immunoblots using a polyclonal antibody to CYP2C29. CYP2C29 is highly homologous to several other CYP2C enzymes and shares the highest amino acid sequence homology with *Cyp2c38*, and *Cyp2c39* (~80-85%) and the lowest with *Cyp2c44* (~60%) (Table 1.1). Knowing the highly homologous nature of murine CYP2C genes, it is likely that this polyclonal antibody would recognize several CYP2C isoforms (Luo et al., 1998; Tsao et al., 2001; DeLozier et al., 2004; Wang et al., 2004). Here in, we hypothesize that: 1) phenytoin and PB induce the murine CYP2C genes, 2) the nuclear receptor CAR mediates PB induction of the murine CYP2C genes, 3) the nuclear receptors CAR or PXR mediate phenytoin induction of the murine CYP2C genes, and 4) that nuclear receptor response elements located in

the 5'-flanking regions of these genes are necessary for phenytoin and PB induction. The specific aims of this study are defined as such:

1- Identify inducible murine CYP2C genes using phenytoin and the prototypical inducer PB.

2- Identify and characterize CAR and PXR nuclear response elements located within the 5'-flanking region of the inducible CYP2C genes

3- Determine whether CAR or PXR mediates phenytoin induction of the inducible CYP2C genes

1.8 References Cited

Asconape JJ (2002) Some common issues in the use of antiepileptic drugs. *Semin Neurol* 22:27-39.

Baes M, Gulick T, Choi HS, Martinoli MG, Simha D and Moore DD (1994) A new orphan member of the nuclear hormone receptor superfamily that interacts with a subset of retinoic acid response elements. *Mol Cell Biol* 14:1544-1552.

Bertilsson G, Heidrich J, Svensson K, Asman M, Jendeberg L, Sydow-Backman M, Ohlsson R, Postlind H, Blomquist P and Berkenstam A (1998) Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. *Proc Natl Acad Sci U S A* 95:12208-12213.

Blumberg B, Sabbagh W, Jr., Juguilon H, Bolado J, Jr., van Meter CM, Ong ES and Evans RM (1998) SXR, a novel steroid and xenobiotic-sensing nuclear receptor. *Genes Dev* 12:3195-3205.

Bohan A and Boyer JL (2002) Mechanisms of hepatic transport of drugs: implications for cholestatic drug reactions. *Semin Liver Dis* 22:123-136.

Campbell WB and Harder DR (1999) Endothelium-derived hyperpolarizing factors and vascular cytochrome P450 metabolites of arachidonic acid in the regulation of tone. *Circ Res* 84:484-488.

Casarett LJ and Doull J (2001) *Casarett and Doull's toxicology : the basic science of poisons*. McGraw-Hill Medical Pub. Division, New York.

Chawla A, Repa JJ, Evans RM and Mangelsdorf DJ (2001) Nuclear receptors and lipid physiology: opening the X-files. *Science* 294:1866-1870.

Chen Y, Ferguson SS, Negishi M and Goldstein JA (2003) Identification of constitutive androstane receptor and glucocorticoid receptor binding sites in the CYP2C19 promoter. *Mol Pharmacol* 64:316-324.

Chen Y, Ferguson SS, Negishi M and Goldstein JA (2004) Induction of human CYP2C9 by rifampicin, hyperforin, and phenobarbital is mediated by the pregnane X receptor. *J Pharmacol Exp Ther* 308:495-501.

Committee (1999) A unified nomenclature system for the nuclear receptor superfamily. *Cell* 97:161-163.

DeLozier TC, Tsao CC, Coulter SJ, Foley J, Bradbury JA, Zeldin DC and Goldstein JA (2004) CYP2C44, a new murine CYP2C that metabolizes arachidonic acid to unique stereospecific products. *J Pharmacol Exp Ther* 310:845-854.

Dussault I, Lin M, Hollister K, Fan M, Termini J, Sherman MA and Forman BM (2002) A structural model of the constitutive androstane receptor defines novel interactions that mediate ligand-independent activity. *Mol Cell Biol* 22:5270-5280.

Ferguson SS, Chen Y, LeCluyse EL, Negishi M and Goldstein JA (2005) Human CYP2C8 is transcriptionally regulated by the nuclear receptors constitutive androstane receptor, pregnane X receptor, glucocorticoid receptor, and hepatic nuclear factor 4alpha. *Mol Pharmacol* 68:747-757.

Ferguson SS, LeCluyse EL, Negishi M and Goldstein JA (2002) Regulation of human CYP2C9 by the constitutive androstane receptor: discovery of a new distal binding site. *Mol Pharmacol* 62:737-746.

Forman BM, Tzameli I, Choi HS, Chen J, Simha D, Seol W, Evans RM and Moore DD (1998) Androstane metabolites bind to and deactivate the nuclear receptor CAR-beta. *Nature* 395:612-615.

Frank C, Gonzalez MM, Oinonen C, Dunlop TW and Carlberg C (2003) Characterization of DNA complexes formed by the nuclear receptor constitutive androstane receptor. *J Biol Chem* 278:43299-43310.

Gerbal-Chaloin S, Daujat M, Pascussi JM, Pichard-Garcia L, Vilarem MJ and Maurel P (2002) Transcriptional regulation of CYP2C9 gene. Role of glucocorticoid receptor and constitutive androstane receptor. *J Biol Chem* 277:209-217.

Gerbal-Chaloin S, Pascussi JM, Pichard-Garcia L, Daujat M, Waechter F, Fabre JM, Carrere N and Maurel P (2001) Induction of CYP2C genes in human hepatocytes in primary culture. *Drug Metab Dispos* 29:242-251.

Goldstein JA (2001) Clinical relevance of genetic polymorphisms in the human CYP2C subfamily. *Br J Clin Pharmacol* 52:349-355.

Goldstein JA and Blaisdell J (1996) Genetic tests which identify the principal defects in CYP2C19 responsible for the polymorphism in mephenytoin metabolism. *Methods Enzymol* 272:210-218.

Goldstein JA and de Morais SM (1994) Biochemistry and molecular biology of the human CYP2C subfamily. *Pharmacogenetics* 4:285-299.

Goodwin B, Hodgson E, D'Costa DJ, Robertson GR and Liddle C (2002) Transcriptional regulation of the human CYP3A4 gene by the constitutive androstane receptor. *Mol Pharmacol* 62:359-365.

Goodwin B, Hodgson E and Liddle C (1999) The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. *Mol Pharmacol* 56:1329-1339.

Goodwin B, Moore LB, Stoltz CM, McKee DD and Kliewer SA (2001) Regulation of the human CYP2B6 gene by the nuclear pregnane X receptor. *Mol Pharmacol* 60:427-431.

Guengerich FP, Wu ZL and Bartleson CJ (2005) Function of human cytochrome P450s: characterization of the orphans. *Biochem Biophys Res Commun* 338:465-469.

Hachad H, Ragueneau-Majlessi I and Levy RH (2002) New antiepileptic drugs: review on drug interactions. *Ther Drug Monit* 24:91-103.

Hankinson O (1995) The aryl hydrocarbon receptor complex. *Annu Rev Pharmacol Toxicol* 35:307-340.

Hodgson E and Levi PE (1997) *A textbook of modern toxicology*. Appleton & Lange, Stamford, C.T.

Hodgson E and Smart RC (2001) *Introduction to biochemical toxicology*. Wiley, New York.

Hoffman EC, Reyes H, Chu FF, Sander F, Conley LH, Brooks BA and Hankinson O (1991) Cloning of a factor required for activity of the Ah (dioxin) receptor. *Science* 252:954-958.

Honkakoski P, Moore R, Washburn KA and Negishi M (1998a) Activation by diverse xenochemicals of the 51-base pair phenobarbital-responsive enhancer module in the CYP2B10 gene. *Mol Pharmacol* 53:597-601.

Honkakoski P and Negishi M (1998) Protein serine/threonine phosphatase inhibitors suppress phenobarbital-induced Cyp2b10 gene transcription in mouse primary hepatocytes. *Biochem J* 330 (Pt 2):889-895.

Honkakoski P and Negishi M (2000) Regulation of cytochrome P450 (CYP) genes by nuclear receptors. *Biochem J* 347:321-337.

Honkakoski P, Zelko I, Sueyoshi T and Negishi M (1998b) The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-

responsive enhancer module of the CYP2B gene. *Mol Cell Biol* 18:5652-5658.

Johnson EF, Palmer CN, Griffin KJ and Hsu MH (1996) Role of the peroxisome proliferator-activated receptor in cytochrome P450 4A gene regulation. *Faseb J* 10:1241-1248.

Johnson EF and Stout CD (2005) Structural diversity of human xenobiotic-metabolizing cytochrome P450 monooxygenases. *Biochem Biophys Res Commun* 338:331-336.

Kawamoto T, Sueyoshi T, Zelko I, Moore R, Washburn K and Negishi M (1999) Phenobarbital-responsive nuclear translocation of the receptor CAR in induction of the CYP2B gene. *Mol Cell Biol* 19:6318-6322.

Kawana K, Ikuta T, Kobayashi Y, Gotoh O, Takeda K and Kawajiri K (2003) Molecular mechanism of nuclear translocation of an orphan nuclear receptor, SXR. *Mol Pharmacol* 63:524-531.

Kliewer SA, Goodwin B and Willson TM (2002) The nuclear pregnane X receptor: a key regulator of xenobiotic metabolism. *Endocr Rev* 23:687-702.

Kliewer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Willson TM, Zetterstrom RH, Perlmann T and Lehmann JM (1998) An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell* 92:73-82.

Kobayashi K, Sueyoshi T, Inoue K, Moore R and Negishi M (2003) Cytoplasmic accumulation of the nuclear receptor CAR by a tetratricopeptide repeat protein in HepG2 cells. *Mol Pharmacol* 64:1069-1075.

Kretschmer XC and Baldwin WS (2005) CAR and PXR: xenosensors of endocrine disrupters? *Chem Biol Interact* 155:111-128.

LeCluyse EL (2001) Pregnane X receptor: molecular basis for species differences in CYP3A induction by xenobiotics. *Chem Biol Interact* 134:283-289.

Lehmann JM, McKee DD, Watson MA, Willson TM, Moore JT and Kliewer SA (1998) The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J Clin Invest* 102:1016-1023.

Liddle C and Goodwin B (2002) Regulation of hepatic drug metabolism: role of the nuclear receptors PXR and CAR. *Semin Liver Dis* 22:115-122.

Luo G, Cunningham M, Kim S, Burn T, Lin J, Sinz M, Hamilton G, Rizzo C, Jolley S, Gilbert D, Downey A, Mudra D, Graham R, Carroll K, Xie J, Madan A, Parkinson A, Christ D, Selling B, LeCluyse E and Gan LS (2002) CYP3A4 induction by drugs: correlation between a pregnane X receptor reporter gene assay and CYP3A4 expression in human hepatocytes. *Drug Metab Dispos* 30:795-804.

Luo G, Zeldin DC, Blaisdell JA, Hodgson E and Goldstein JA (1998) Cloning and expression of murine CYP2Cs and their ability to metabolize arachidonic acid. *Arch Biochem Biophys* 357:45-57.

Maglich JM, Stoltz CM, Goodwin B, Hawkins-Brown D, Moore JT and Kliewer SA (2002) Nuclear pregnane x receptor and constitutive androstane receptor regulate overlapping but distinct sets of genes involved in xenobiotic detoxification. *Mol Pharmacol* 62:638-646.

Makinen J, Frank C, Jyrkkarinne J, Gynther J, Carlberg C and Honkakoski P (2002) Modulation of mouse and human phenobarbital-responsive enhancer module by nuclear receptors. *Mol Pharmacol* 62:366-378.

Makishima M (2005) Nuclear receptors as targets for drug development: regulation of cholesterol and bile acid metabolism by nuclear receptors. *J Pharmacol Sci* 97:177-183.

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P and Evans RM (1995) The nuclear receptor superfamily: the second decade. *Cell* 83:835-839.

Meyer RP, Hagemeyer CE, Knoth R, Kurz G and Volk B (2001a) Oxidative hydrolysis of scoparone by cytochrome p450 CYP2C29 reveals a novel metabolite. *Biochem Biophys Res Commun* 285:32-39.

Meyer RP, Knoth R, Schiltz E and Volk B (2001b) Possible function of astrocyte cytochrome P450 in control of xenobiotic phenytoin in the brain: in vitro studies on murine astrocyte primary cultures. *Exp Neurol* 167:376-384.

Miners JO and Birkett DJ (1998) Cytochrome P4502C9: an enzyme of major importance in human drug metabolism. *Br J Clin Pharmacol* 45:525-538.

Moore DD (2005) CAR: three new models for a problem child. *Cell Metab* 1:6-8.

Moore JT, Moore LB, Maglich JM and Kliewer SA (2003) Functional and structural comparison of PXR and CAR. *Biochim Biophys Acta* 1619:235-238.

Moore LB, Maglich JM, McKee DD, Wisely B, Willson TM, Kliewer SA, Lambert MH and Moore JT (2002) Pregnane X receptor (PXR), constitutive androstane receptor (CAR), and benzoate X receptor (BXR) define three pharmacologically distinct classes of nuclear receptors. *Mol Endocrinol* 16:977-986.

Moore LB, Parks DJ, Jones SA, Bledsoe RK, Consler TG, Stimmel JB, Goodwin B, Liddle C, Blanchard SG, Willson TM, Collins JL and Kliewer SA (2000) Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. *J Biol Chem* 275:15122-15127.

Nebert DW and Jones JE (1989) Regulation of the mammalian cytochrome P1-450 (CYP1A1) gene. *Int J Biochem* 21:243-252.

Nelson DR (1999) Cytochrome P450 and the individuality of species. *Arch Biochem Biophys* 369:1-10.

Neuhold LA, Shirayoshi Y, Ozato K, Jones JE and Nebert DW (1989) Regulation of mouse CYP1A1 gene expression by dioxin: requirement of two cis-acting elements during induction. *Mol Cell Biol* 9:2378-2386.

Node K, Huo Y, Ruan X, Yang B, Spiecker M, Ley K, Zeldin DC and Liao JK (1999) Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. *Science* 285:1276-1279.

Okey AB (1990) Enzyme induction in the cytochrome P-450 system. *Pharmacol Ther* 45:241-298.

Okey AB, Bondy GP, Mason ME, Kahl GF, Eisen HJ, Guenthner TM and Nebert DW (1979) Regulatory gene product of the Ah locus. Characterization of the cytosolic inducer-receptor complex and evidence for its nuclear translocation. *J Biol Chem* 254:11636-11648.

Pascussi JM, Gerbal-Chaloin S, Drocourt L, Assenat E, Larrey D, Pichard-Garcia L, Vilarem MJ and Maurel P (2004) Cross-talk between xenobiotic detoxication and other signalling pathways: clinical and toxicological consequences. *Xenobiotica* 34:633-664.

Pascussi JM, Gerbal-Chaloin S, Drocourt L, Maurel P and Vilarem MJ (2003) The expression of CYP2B6, CYP2C9 and CYP3A4 genes: a tangle of networks of nuclear and steroid receptors. *Biochim Biophys Acta* 1619:243-253.

Poland A and Glover E (1974) Comparison of 2,3,7,8-tetrachlorodibenzo-p-dioxin, a potent inducer of aryl hydrocarbon hydroxylase, with 3-methylcholanthrene. *Mol Pharmacol* 10:349-359.

Poland A, Glover E and Kende AS (1976) Stereospecific, high affinity binding of 2,3,7,8-tetrachlorodibenzo-p-dioxin by hepatic cytosol. Evidence that the binding species is receptor for induction of aryl hydrocarbon hydroxylase. *J Biol Chem* 251:4936-4946.

Raucy JL (2003) Regulation of CYP3A4 expression in human hepatocytes by pharmaceuticals and natural products. *Drug Metab Dispos* 31:533-539.

Raucy JL, Mueller L, Duan K, Allen SW, Strom S and Lasker JM (2002) Expression and induction of CYP2C P450 enzymes in primary cultures of human hepatocytes. *J Pharmacol Exp Ther* 302:475-482.

Reyes H, Reisz-Porszasz S and Hankinson O (1992) Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor. *Science* 256:1193-1195.

Rowlands JC and Gustafsson JA (1997) Aryl hydrocarbon receptor-mediated signal transduction. *Crit Rev Toxicol* 27:109-134.

Shan L, Vincent J, Brunzelle JS, Dussault I, Lin M, Ianculescu I, Sherman MA, Forman BM and Fernandez EJ (2004) Structure of the murine constitutive androstane receptor complexed to androstenol: a molecular basis for inverse agonism. *Mol Cell* 16:907-917.

Sharma R, Lake BG and Gibson GG (1988) Co-induction of microsomal cytochrome P-452 and the peroxisomal fatty acid beta-oxidation pathway in the rat by clofibrate and di-(2-ethylhexyl)phthalate. Dose-response studies. *Biochem Pharmacol* 37:1203-1206.

Shimada T, Yamazaki H, Mimura M, Inui Y and Guengerich FP (1994) Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *J Pharmacol Exp Ther* 270:414-423.

Squires EJ, Sueyoshi T and Negishi M (2004) Cytoplasmic localization of pregnane X receptor and ligand-dependent nuclear translocation in mouse liver. *J Biol Chem* 279:49307-49314.

Staudinger J, Liu Y, Madan A, Habeebu S and Klaassen CD (2001a) Coordinate regulation of xenobiotic and bile acid homeostasis by pregnane X receptor. *Drug Metab Dispos* 29:1467-1472.

Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, Reinhard J, Willson TM, Koller BH and Kliewer SA (2001b) The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc Natl Acad Sci U S A* 98:3369-3374.

Sueyoshi T, Kawamoto T, Zelko I, Honkakoski P and Negishi M (1999) The repressed nuclear receptor CAR responds to phenobarbital in activating the human CYP2B6 gene. *J Biol Chem* 274:6043-6046.

Sueyoshi T and Negishi M (2001) Phenobarbital response elements of cytochrome P450 genes and nuclear receptors. *Annu Rev Pharmacol Toxicol* 41:123-143.

Suino K, Peng L, Reynolds R, Li Y, Cha JY, Repa JJ, Kliewer SA and Xu HE (2004) The nuclear xenobiotic receptor CAR: structural determinants of constitutive activation and heterodimerization. *Mol Cell* 16:893-905.

Swales K and Negishi M (2004) CAR, driving into the future. *Mol Endocrinol* 18:1589-1598.

Thummel KE and Wilkinson GR (1998) In vitro and in vivo drug interactions involving human CYP3A. *Annu Rev Pharmacol Toxicol* 38:389-430.

Tsao CC, Coulter SJ, Chien A, Luo G, Clayton NP, Maronpot R, Goldstein JA and Zeldin DC (2001) Identification and localization of five CYP2Cs in murine extrahepatic tissues and their metabolism of arachidonic acid to regio- and stereoselective products. *J Pharmacol Exp Ther* 299:39-47.

Tzameli I, Pissios P, Schuetz EG and Moore DD (2000) The xenobiotic compound 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene is an agonist ligand for the nuclear receptor CAR. *Mol Cell Biol* 20:2951-2958.

Van Allen MW (1984) Landmark perspective: Introduction of sodium diphenyl hydantoinate. *Jama* 251:1068-1069.

Wang H, Faucette S, Sueyoshi T, Moore R, Ferguson S, Negishi M and LeCluyse EL (2003a) A novel distal enhancer module regulated by pregnane X receptor/constitutive androstane receptor is essential for the maximal induction of CYP2B6 gene expression. *J Biol Chem* 278:14146-14152.

Wang H, Faucette SR, Gilbert D, Jolley SL, Sueyoshi T, Negishi M and LeCluyse EL (2003b) Glucocorticoid receptor enhancement of pregnane X receptor-mediated CYP2B6 regulation in primary human hepatocytes. *Drug Metab Dispos* 31:620-630.

Wang H, Zhao Y, Bradbury JA, Graves JP, Foley J, Blaisdell JA, Goldstein JA and Zeldin DC (2004) Cloning, expression, and characterization of three new mouse cytochrome p450 enzymes and partial characterization of their fatty acid oxidation activities. *Mol Pharmacol* 65:1148-1158.

Watkins RE, Wisely GB, Moore LB, Collins JL, Lambert MH, Williams SP, Willson TM, Kliewer SA and Redinbo MR (2001) The human nuclear xenobiotic receptor PXR: structural determinants of directed promiscuity. *Science* 292:2329-2333.

Waxman DJ (1999) P450 gene induction by structurally diverse xenochemicals: central role of nuclear receptors CAR, PXR, and PPAR. *Arch Biochem Biophys* 369:11-23.

Wei P, Zhang J, Dowhan DH, Han Y and Moore DD (2002) Specific and overlapping functions of the nuclear hormone receptors CAR and PXR in xenobiotic response. *Pharmacogenomics J* 2:117-126.

Wei P, Zhang J, Egan-Hafley M, Liang S and Moore DD (2000) The nuclear receptor CAR mediates specific xenobiotic induction of drug metabolism. *Nature* 407:920-923.

Yoshinari K, Kobayashi K, Moore R, Kawamoto T and Negishi M (2003) Identification of the nuclear receptor CAR:HSP90 complex in mouse liver and recruitment of protein phosphatase 2A in response to phenobarbital. *FEBS Lett* 548:17-20.

Zhang J, Huang W, Qatanani M, Evans RM and Moore DD (2004) The constitutive androstane receptor and pregnane X receptor function coordinately to prevent bile acid-induced hepatotoxicity. *J Biol Chem* 279:49517-49522.

Zou AP, Drummond HA and Roman RJ (1996) Role of 20-HETE in elevating loop chloride reabsorption in Dahl SS/Jr rats. *Hypertension* 27:631-635.

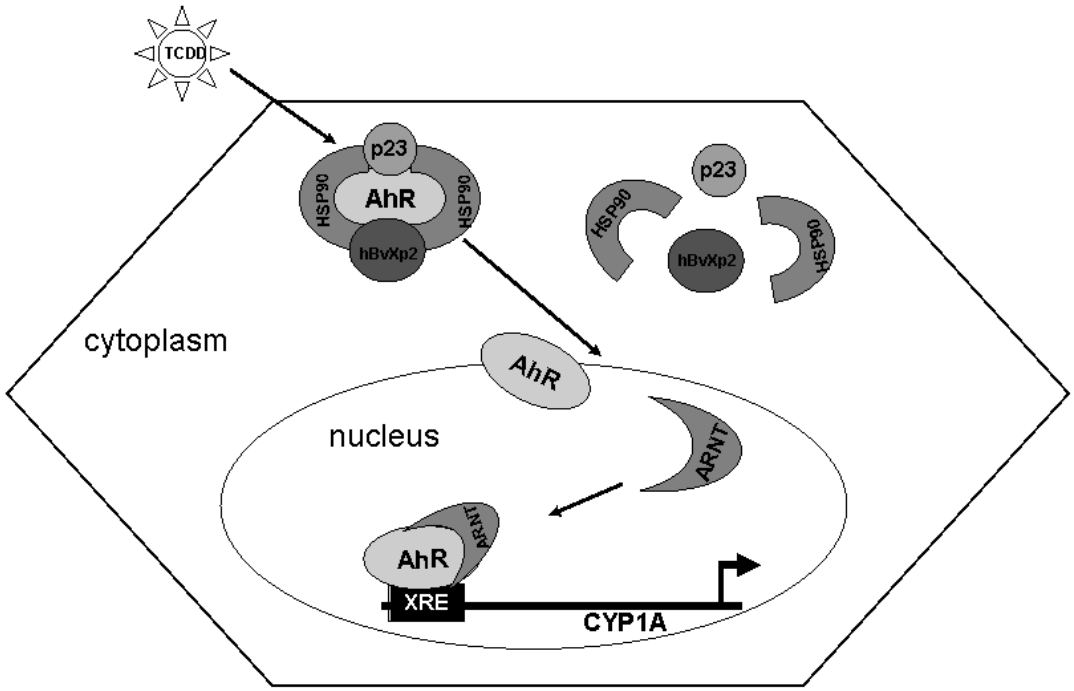

Figure 1.1- Molecular mechanism of AhR mediated transcriptional activation. An inactive AhR is located in the cytosol associated with two heat shock proteins (HSP90), the co-chaperone p23, and the hepatitis B virus X-associated protein 2 (Pascussi et al., 2004).

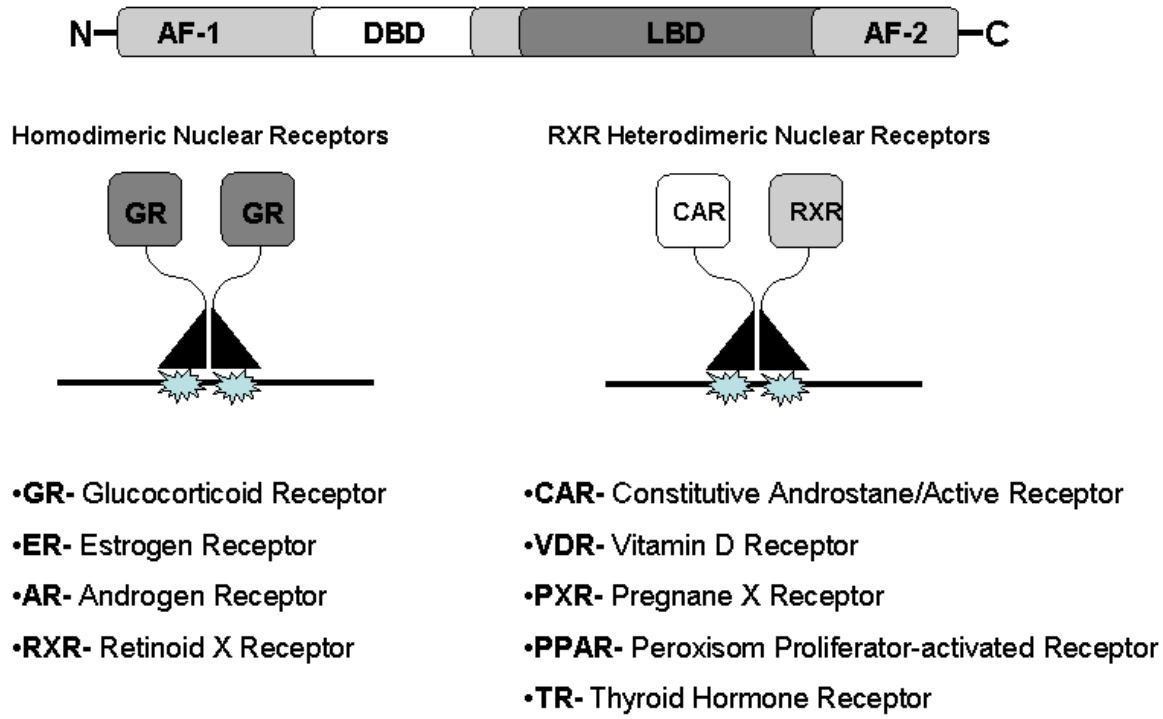

Figure 1.2- Nuclear Receptors share a common structure and are known to form both homodimers and heterodimers. Shown are representative receptors for each group (Mangelsdorf et al., 1995; Moore et al., 2002; Moore et al., 2003; Pascussi et al., 2004).

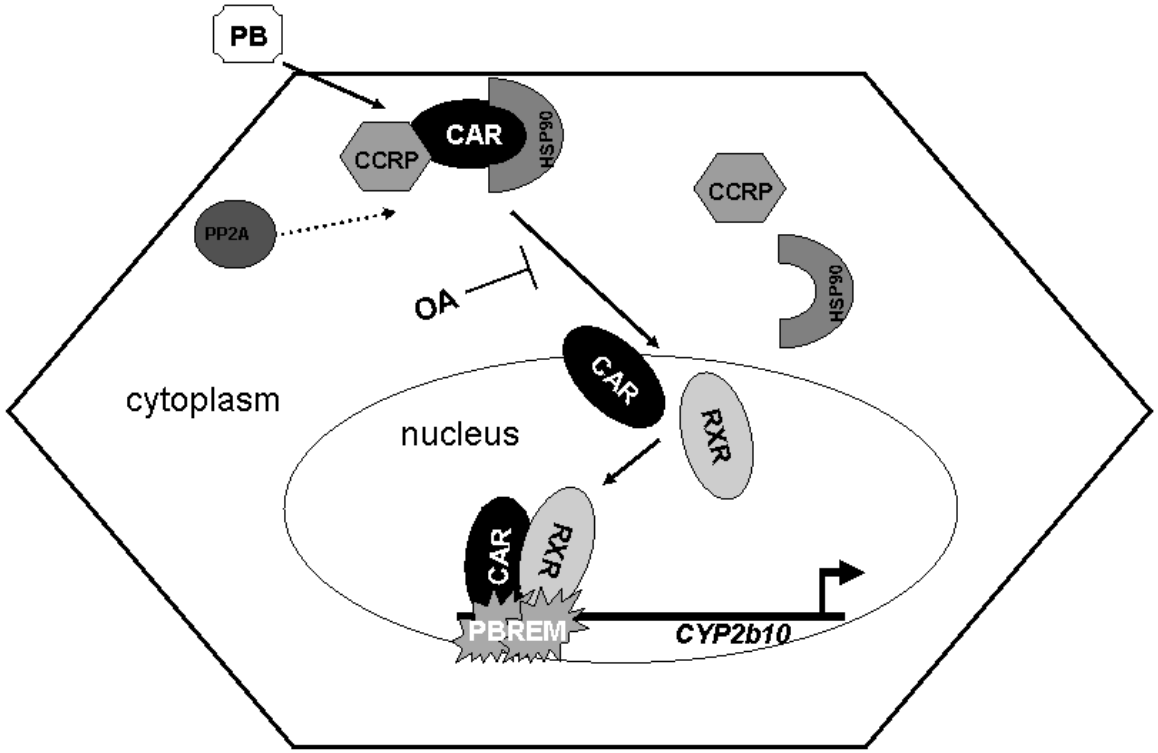

Figure 1.3- Molecular mechanism of CAR mediated transcriptional activation. Inactive CAR is sequestered in the cytosol in association with CCRP and HSP90. PB treatment initiates the nuclear translocation of CAR. Phosphorylation is thought to be involved in the nuclear translocation of CAR because the phosphatase inhibitor okidiac acid (OA) prevents the nuclear accumulation of CAR. PP2A has been shown to be associated with CAR-CCRP-HSP90 protein complex after PB treatment (Sueyoshi and Negishi, 2001).

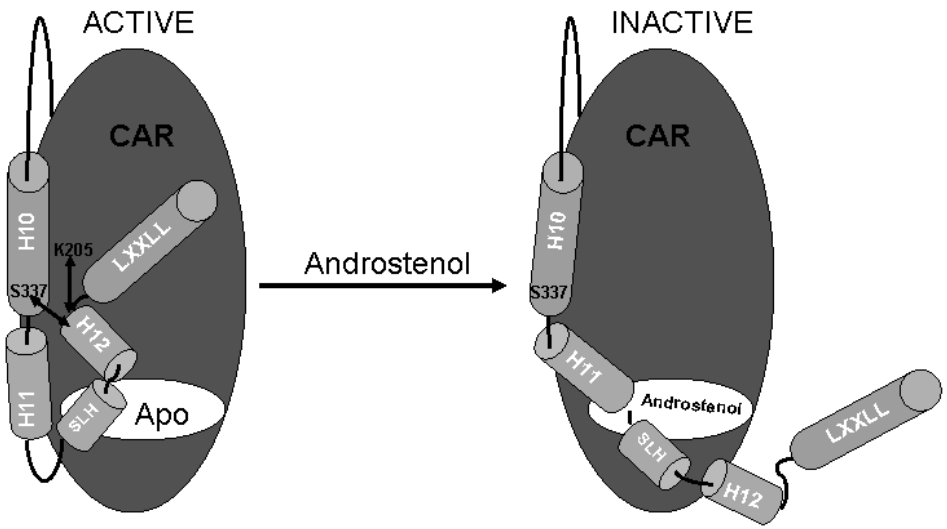

Figure 1.4- Illustration representing the transition of active CAR to inactive CAR by the binding of androstenol. The short linker helix (SLH) and the hydrogen bonding interactions of helix 12 with helix 4 and helix 10 stabilize the AF-2 domain of CAR in the active confirmation. The binding of androstenol inhibits the constitutive activity of CAR by introducing a kink in helix 10 at S337, which results in the displacement of the SLH and disrupts the hydrogen bonding interactions of helix 12. This illustration was based upon original drawing by Moore (2005).

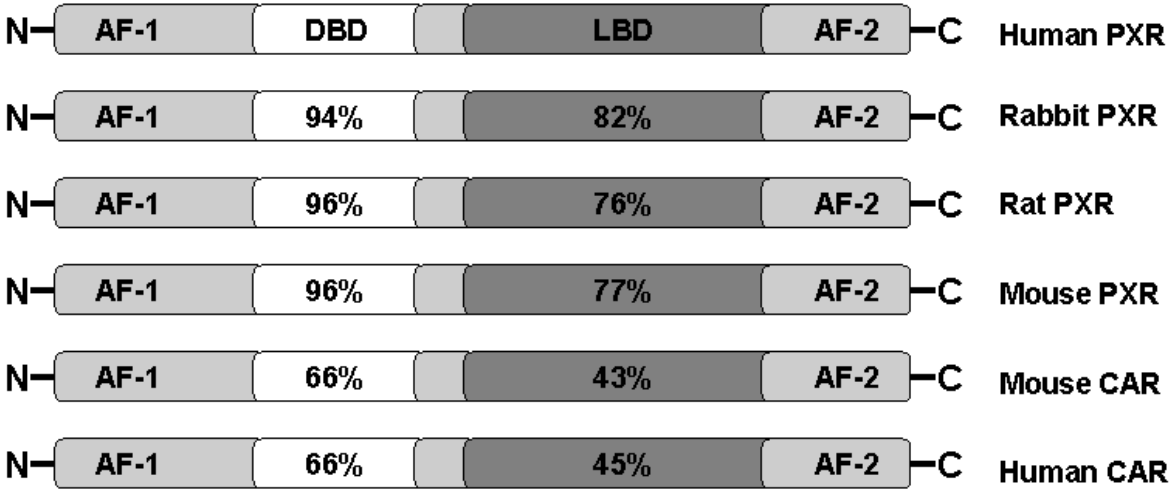

Figure 1.5- Sequence comparison of PXR and CAR across species. Similarities in the DBD and LBD are expressed as percentage amino acid identity (Kliewer et al., 2002; Moore et al., 2002; Moore et al., 2003).

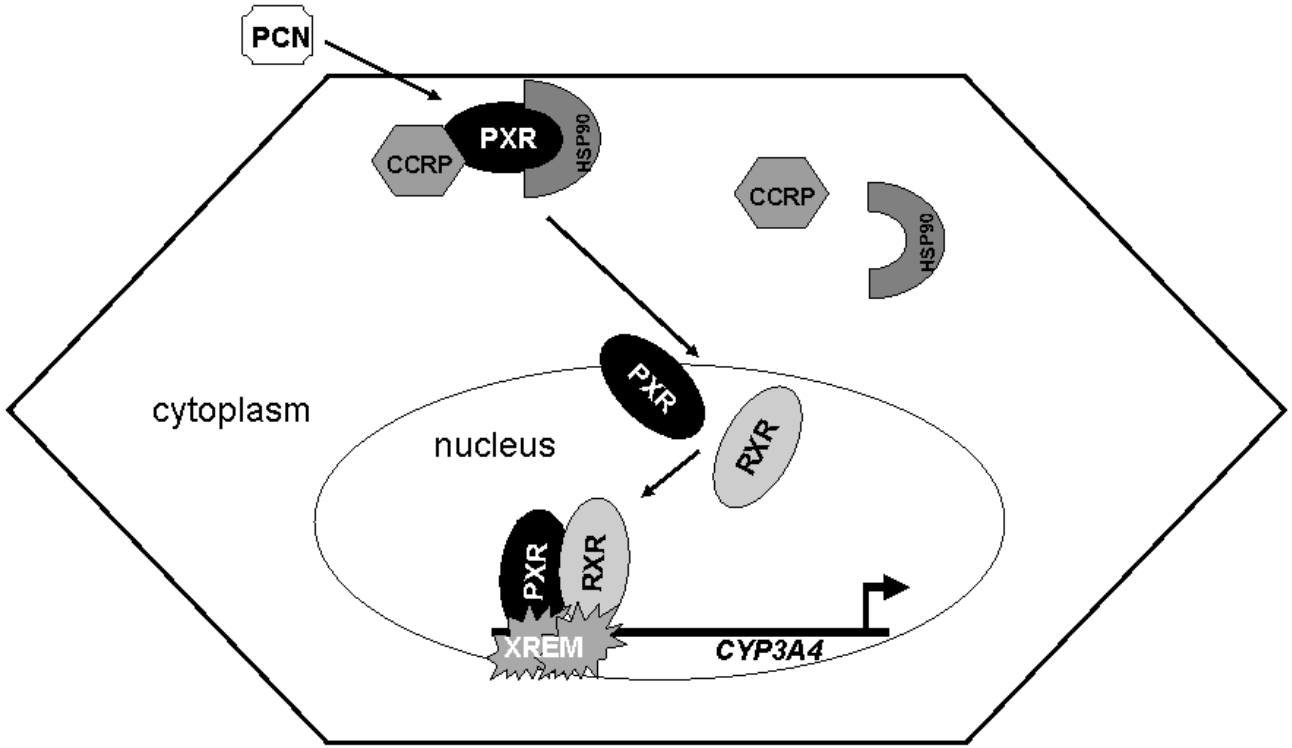

Figure 1.6- Molecular mechanism of PXR mediated transcriptional activation. PXR has been shown to be sequestered in the cytosol associated with CCRP and HSP90 (Kliewer et al., 2002; Pascussi et al., 2004; Squires et al., 2004) PXR is activated by ligand binding and subsequently translocates to the nucleus to activate gene transcription.

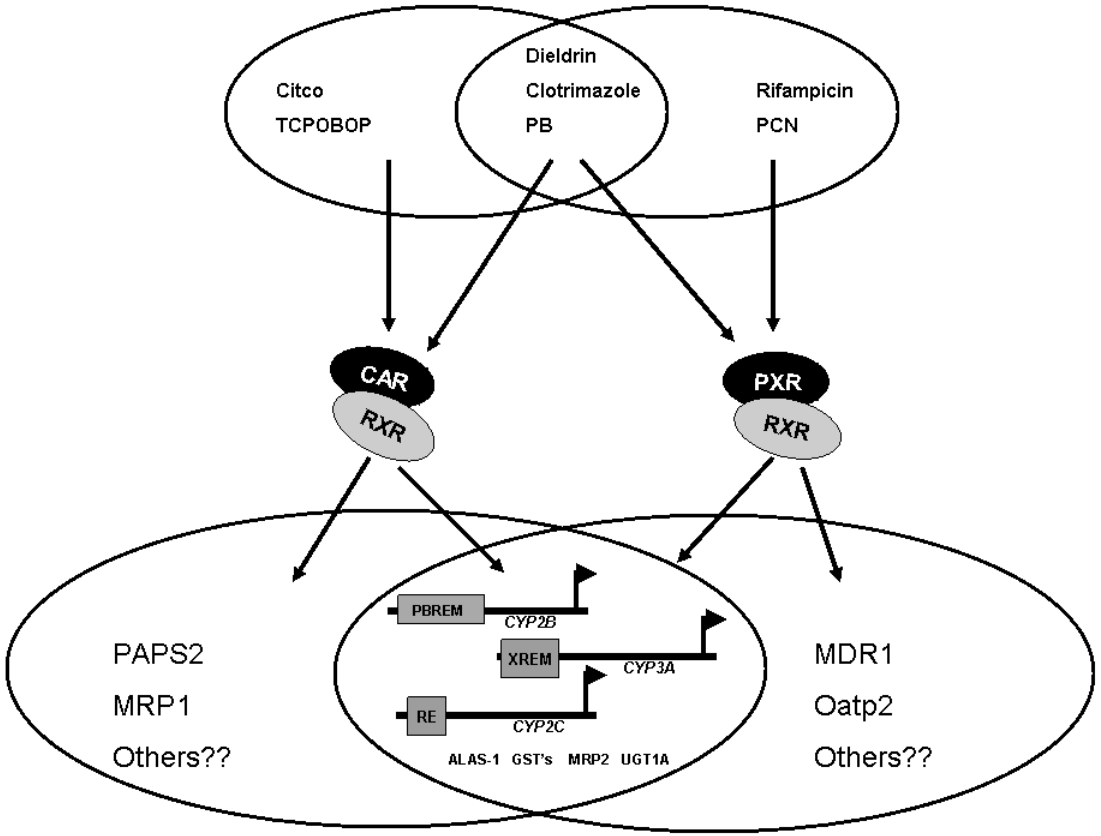

Figure 1.7- An illustration depicting the observed cross-talk between the distinct signaling pathways of CAR and PXR. CAR and PXR have been shown to share some activators with the caveat that species differences are observed. Similarly, CAR and PXR have been shown to regulate many of the same drug metabolizing and transporter genes using the same response elements (Honkakoski and Negishi, 2000; Kliewer et al., 2002; Maglich et al., 2002; Pascussi et al., 2003; Pascussi et al., 2004).

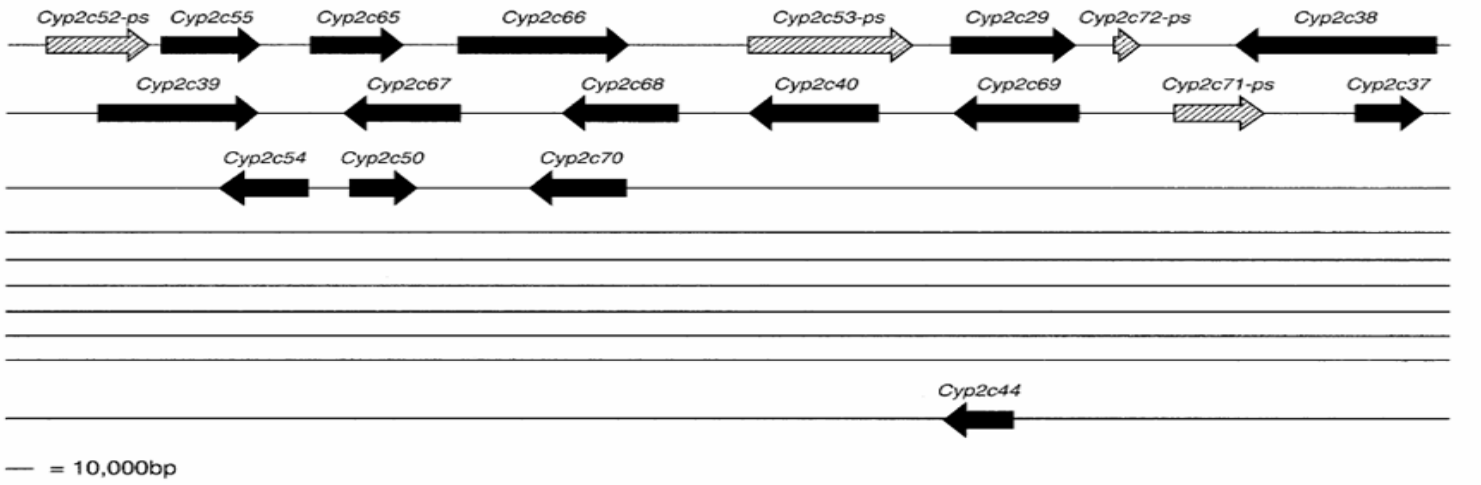

Figure 1.8- Illustration depicting the organization of the murine CYP2C genes on chromosome 19 (GA_x6K02T2RE5P:29000001..34500000). Exonic sequences of the known mouse, rat, and human CYP2C subfamily members were used to search the mouse genomic sequences in the Celera Discovery System. Fifteen murine Cyp2c genes (solid arrows) and 4 Cyp2c pseudogenes (hatched arrows) were identified. All of these genes were located within a 1.5 Mb cluster (shown proximal to distal), except for Cyp2c44, which is located 3.8 Mb downstream. Illustration originally published by Wang et al. (2004)

Table 1.1 Amino acid identity among several members of the murine CYP2C subfamily (Luo et al., 1998; DeLozier et al., 2004; Wang et al., 2004).

	Cyp2c29	**Cyp2c37**	**Cyp2c38**	**Cyp2c39**	**Cyp2c40**	**Cyp2c44**	**Cyp2c50**	**Cyp2c54**	**Cyp2c55**
Cyp2c29		77	84	85	69	60	77	76	71
Cyp2c37	77		74	75	71	60	94	91	70
Cyp2c38	84	74		92	70	58	75	74	70
Cyp2c39	85	75	92		70	59	76	74	70
Cyp2c40	69	71	70	70		52	71	70	65
Cyp2c44	60	60	58	59	52		59	58	58
Cyp2c50	77	94	75	76	71	59		92	67
Cyp2c54	76	91	74	74	70	58	92		67
Cyp2c55	71	70	70	70	65	58	67	67	

2. THE CONSTITUTIVE ACTIVE/ANDROSTANE RECEPTOR REGULATES PHENYTOIN INDUCTION OF *Cyp2C29*

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This manuscript was published June 2004 in Molecular Pharmacology 65:1397-1404

2.1 Abstract

Many cytochrome P450 isoforms are known to be drug-inducible. The anticonvulsant, phenytoin, has been reported to be an inducer of human CYP2B6, CYP3A4 and murine CYP2C29. However, the molecular mechanism mediating phenytoin induction remains unclear. Herein, we utilized *in vivo* and *in vitro* gene reporter assays of the *Cyp2c29* promoter to delineate the phenytoin-response activity to a phenytoin responsive module (PHREM) located -1371 bp upstream of the *Cyp2c29* translation start site. The PHREM, consisting of two motifs of imperfect direct repeat hexamers spaced by four nucleotides and a putative CCAAT/enhancer-binding protein-binding site, mediated luciferase reporter induction by phenytoin in mouse livers *in vivo* and was activated by CAR in HepG2 cells. Hepatic CYP2C29 mRNA and protein was induced by phenytoin in wild-type mice. However, phenytoin induction of CYP2C29 mRNA was removed in CAR-null mice, indicating that CAR regulates phenytoin-induced transcription of the *Cyp2c29* gene. Furthermore, the constitutive levels of CYP2C29 mRNA were reduced ~77 fold in CAR-null mice compared with those in the wild-type mice, suggesting that CAR may also regulate the constitutive expression of the *Cyp2c29* gene either directly or indirectly.

2.2 Introduction

P450s are a superfamily of heme-containing monooxygenases that metabolize a wide range of endogenous and exogenous compounds. Regulation of P450 expression is a major source of variability in the metabolism of xenobiotics and endogenous compounds. Induction of P450 expression can lead to alterations in drug efficacy and create the potential for drug-drug interactions (Honkakoski and Negishi, 2000; Sueyoshi and Negishi, 2001). Drug induction of P450s is often regulated by so-called xenobiotic sensing nuclear receptors such as CAR, pregnane X receptor (PXR) and peroxisome proliferator-activated receptor (PPAR). While phenytoin is reported to induce the *Cyp2c29 gene* (Meyer et al., 2001a; Meyer et al., 2001b), the phenytoin response element and the nuclear receptor that regulate the element remain unidentified.

Phenytoin and PB are known to be inducers of several P450 isoforms. PB is the prototypical inducer for a structurally diverse group of chemicals, which increase the expression of several isoforms in the CYP2B and CYP2C subfamilies (Honkakoski et al., 1998a; Sueyoshi, 1999; Sueyoshi and Negishi, 2001). PB induction of *Cyp2b10* is regulated by CAR (Honkakoski et al., 1998b; Honkakoski and Negishi, 2000; Sueyoshi and Negishi, 2001). In mouse hepatocytes or in mouse liver *in vivo*, CAR is predominately sequestered in the cytoplasm and translocates into the nucleus after treatment with PB (Honkakoski and Negishi, 2000; Sueyoshi and Negishi, 2001; Kobayashi et al., 2003; Yoshinari et al., 2003). In the nucleus, CAR forms heterodimers with the retinoid X receptor (RXR) and binds to enhancer elements such as the phenobarbital responsive enhancer module

(PBREM) in the mouse *Cyp2b10* and human *CYP2B6* genes (Honkakoski et al., 1998b; Sueyoshi, 1999; Honkakoski and Negishi, 2000; Sueyoshi and Negishi, 2001). The nuclear receptor binding motifs within the PBREM are organized into two imperfect direct repeat hexamers spaced by four nucleotides (DR-4) (Honkakoski et al., 1998a; Honkakoski et al., 1998b). Related DR-4 binding motifs, capable of binding CAR, are also found in other genes such as human *CYP3A4* and *CYP2C9* (Honkakoski and Negishi, 2000; Sueyoshi and Negishi, 2001; Ferguson et al., 2002; Gerbal-Chaloin et al., 2002; Goodwin, 2002).

Unlike CAR, PXR requires ligand binding for activation (Kliewer et al., 2002). Prototypical ligands of PXR increase the expression of *CYP3A4* (Honkakoski and Negishi, 2000; Sueyoshi and Negishi, 2001; Kast et al., 2002; Luo et al., 2002). Once activated, PXR forms heterodimers with RXR and binds enhancer elements such as the xenobiotic responsive enhancer module (XREM) within the regulatory region of the human *CYP3A4* gene (Honkakoski and Negishi, 2000; Sueyoshi and Negishi, 2001; Liddle and Goodwin, 2002). The XREM is composed of two nuclear receptor-binding motifs spaced by 29 nucleotides, a DR-3 and an imperfect everted hexamer repeat spaced by 6 nucleotides (ER-6). Although the XREM is considered to be the archetypical PXR binding site, the DNA binding preferences of PXR and CAR are known to be quite similar (Honkakoski and Negishi, 2000; Makinen et al., 2002).

Recently, the epileptic drug phenytoin was found to induce CYP3A4 and CYP2B6 mRNA in primary human hepatocytes (Luo et al., 2002; Raucy, 2003; Wang et al., 2003). Raucy (2003) showed that phenytoin produced a marked

increase in CYP3A4 mRNA, equivalent to that seen with rifampicin. Surprisingly, using a *CYP*3A4 XREM luciferase promoter construct in HepG2 cells transfected with human PXR, promoter activation by phenytoin was much weaker than that produced by rifampicin (Luo et al., 2002; Raucy, 2003).

A recent study suggested that phenytoin and PB induce CYP2C29, the major murine CYP2C, protein in liver microsomes (Meyer et al., 2001a; Meyer et al., 2001b). However, this preliminary conclusion was based on Western blot data with a polyclonal antibody to CYP2C29. Knowing the highly homologous nature of murine CYP2C29 to the four other published members of this subfamily (84-85% similar to CYP2C38 and CYP2C39), it is likely that this polyclonal antibody would recognize several CYP2C isoforms (Luo et al., 1998). Due to the recent interest in phenytoin as an inducer of P450 enzymes and the lack of conclusive evidence indicating whether this induction is mediated by CAR or PXR, the murine model provides an excellent system to investigate the molecular mechanism of this induction event. The goals of the present study were to determine whether transcription of the *Cyp2c29* gene is inducible by phenytoin and PB, to identify putative nuclear receptor responsive elements within the upstream promoter region, to examine putative element function using wild-type and mutant *Cyp2c29* luciferase promoter constructs, and to determine whether CAR mediates induction of murine *Cyp2c29* by phenytoin and PB using CAR-null mice.

2.3 Materials and Methods

Materials and Reagents

PB (sodium salt), phenytoin (5, 5-diphenylhydantoin, sodium salt) and 5α-androstenol were purchased from Sigma-Aldrich (St. Louis, MO). DMSO and other common reagents not listed were also purchased from Sigma-Aldrich or standard sources. 1,4-bis[2-(3,5-Dichloropyridyloxy)]benzene (TCPOBOP) was a kind gift from Dr. James Sideway (AstraZeneca). Cell culture media, fetal bovine serum, and trypsin/EDTA were purchased from Invitrogen (Carlsbad, CA). Penicillin and streptomycin were purchased from Sigma-Aldrich. Oligonucleotides were purchased from Genosys, Inc. (The Woodlands, TX) at 50 μM scale and desalted. HepG2 cells were purchased from American Type Culture Collection. (Manassas, VA)

Isolation of Total RNA and Quantitative RT-PCR Analysis

Total RNA was extracted using an ABI 6100 Nucleic Acid PrepStation. All chemicals for the ABI 6100 were purchased from Applied Biosystems (Foster City, CA). Total RNA was isolated and stored at -80^{o}C individually. Prior to reverse transcription, a portion of each total RNA isolate was pooled within each experimental group. Quantitative RT-PCR analysis was performed in two steps by an initial reaction with MuLV Reverse Transcriptase (Applied Biosystems), followed by PCR using 2X SYBR Green Master Mix . Reverse transcription was performed with 120 ng of total RNA combined with a buffer containing 0.4 μl (8 units) of Rnase Inhibitor (Applied Biosystems), 1x PCR Buffer II, 5.5 mM $MgCl_2$, 0.5 mM dATP, dCTP, dTTP, and dGTP (each), 2.5 μM random hexamers, and 0.5 μl (25 units) of

MuLV Reverse Transcriptase in a final volume of 20µl. Reverse transcription reactions were incubated using a PCR System 9700 Thermocycler (Applied Biosystems) using the following cycling parameters: 25^{o}C for 10 min, 42^{o}C for 60 min, 95^{o}C for 5 min (inactivation), and 4^{o}C hold. Quantitative PCR was performed on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). PCR reaction consisted of 2 µl of cDNA template, 1X SYBR Green Buffer Master Mix and 5 pmol of each primer in a final volume of 20 µl. Sequences of specific primers for CYP2B10, CYP2C29, and β-actin are shown in Table 2.1. Quantitative PCR parameters for cycling were as follows: 50^{o}C for 2 min hold, 95^{o}C for 10 min hold, denaturation at 94^{o}C for 30 sec for 42 cycles, annealing at 60^{o}C (CYP2B10 & β-actin primers) or at 55^{o}C (CYP2C29 primers) for 30 sec for 42 cycles, and 72^{o}C extension for 30 sec for 42 cycles. PCR products were determined to be single products for each gene specific primer set using 3% agarose gels stained with ethidium bromide and dissociation curve analyses followed by dye terminator DNA sequencing (Applied Biosystems). Standard curves (Log of template dilution vs Ct value) for each gene specific primer set were used to determine relative mRNA content for each target gene.

Plasmids and Cloning of Cyp2c29 Promoter Region

pGL3-Basic vector was purchased from Promega (Madison, WI). Author M.N. provided nuclear receptor expression plasmids pCR3-mCAR and pSG5-hPXR. PCR was performed to isolate the *Cyp2c29* -1.5 kb upstream promoter region using mouse genomic DNA as the template (Promega Madison, WI). Primers 52F and

53R (Table 2.1) were used to amplify the *Cyp2c29* -1.5 kb promoter region. Primer 52F introduced an *Xho* I restriction site 5' and primer 53R introduced an *Nco* I site 3'. The isolated amplicon was subcloned into pCRTopoTA vector (Invitrogen). After sequence verification with genomic sequence data, the *Xho* I and *Nco* I were used to remove the *Cyp2c29* -1.5 kb promoter region and insert it into the pGL3-Basic vector. Mutant *Cyp2c29* 1.5 kb promoter constructs were produced by site-directed mutagenesis using QuikChange (Stratagene, La Jolla, CA). Mutants were sequenced to ensure that only the intended nucleotides were changed. Subsequently the 1.5 kb promoter region was removed and inserted into fresh pGL3Basic vector at *Xho* I and *Nco* I sites. Mutant oligonucleotides used in mutagenesis reactions are shown in Table 2.2 (mutated nucleotides are in bold). Dye terminator DNA sequencing (Applied Biosystems) was used to verify all sequences.

Culture and Transfection of HepG2 Cells

HepG2 cells were cultured in Eagle's minimal essential medium (MEM) with 30mg/L penicillin and 50mg/L streptomycin and supplemented with 10% fetal bovine serum. Transfections were performed with Effectene transfection reagent (Qiagen, Valencia, CA) using the manufacturer's recommended procedures. Cells were plated in 24-well plates and transfected for 12 to 18 hrs before drug treatment.

Transcriptional Activation Assay

HepG2 cells were maintained in Eagle's MEM with 10% fetal bovine serum. Cells were passaged at a density of approximately 100,000 cells/well into 24 well plates. Transfections typically included 100 ng of receptor, 100 ng of reporter, and 1 ng of pRL-tk transfection control. Twelve to 18 hrs after transfection, cells were washed per manufacturer's recommendations, treated with drugs or vehicle and incubated for 24 hrs. Cells were subsequently lysed with 100 µl of passive lysis buffer (Promega) for 30 min at room temp with gentle rocking, and dual luciferase assays (Promega) were then performed on cell lysates per the manufacturer's procedures.

Gel Mobility Shift Assays

Electrophoretic mobility shift assays (EMSA) were performed by methods analogous to the procedure described by Ferguson (2002). Briefly, human RXR, mouse CAR, and human PXR were synthesized *in vitro* using the TNT quick-coupled *in vitro* transcription/translation system (Promega) following the manufacturer's protocol. Oligonucleotides (Table 2) were labeled with [γ-^{32}P]dCTP and the probe was purified by Microspin G-25 columns (Amersham Biosciences, Piscataway, NJ). A volume of labeled probe (~35,000 cpm per rxn) was applied to each binding reaction in 10 mM HEPES (pH 7.6), 0.5 mM dithiothreitol, 15% glycerol, 0.05% Nonidet p-40, 50 mM NaCl, 2 µg of poly(dI-dC), and 1 µl of *in vitro* transcribed/translated proteins in a final volume of 10 µl. The reactions were incubated at room temperature for 20 min after addition of probe, then loaded on a

5% acrylamide gel in Tris/acetate/EDTA buffer, dried, and exposed to film for 6 to 18 hrs at -70^{o}C.

Animals

Male C57BL/6NCrlBR (C57BL/6) and C3H/HeNCrlBR (C3H) mice were purchased from Charles Rivers Laboratory (Wilmington, MA). A CAR-null mouse (Ueda et al., 2002) was first crossbred with C3H to generate CAR heterozygous offspring. Subsequently, CAR heterozygous offspring were repeatedly backcrossed with C3H mice until the genetic background became over 95% C3H. The obtained heterozygous mice were bred to produce the wild-type and CAR-null C3H mice. Mice were fed with a standard solid diet and tap water *ad libitum* for 5 days. Animals received corn oil (vehicle), PB (80 mg/kg), or phenytoin (80 mg/kg) via oral gavage at a volume of 10 ml/kg for 4 days. Animals were sacrificed on the fifth day and the livers removed for total RNA isolation or luciferase activity quantitation. The NIEHS committee for the humane care and treatment of research animals approved all animal procedures.

Tail Vein Injections

Mice were injected using the Mirus *in vivo* gene delivery system (Madison, WI). Injections consisted of 6 μg of the *Cyp*2c29 promoter reporter along with 4 μg of pRL-tk transfection control reporter and Mirus proprietary polymers. Injections were performed following the manufacturer's suggested procedure on the fourth day

of drug treatment. Animals were sacrificed next day and livers were removed for quantitation of luciferase activity.

Western Blot Analysis

Mice were treated intraperitoneally with vehicle (DMSO, 10 ml/kg), PB (80 mg/kg), or phenytoin (80 mg/kg). Livers were removed 3 hours after treatment and nuclear extracts were prepared as previously described (Sueyoshi et al., 1995). Immunoblotting of the nuclear extract was performed as previously described (Honkakoski et al., 1998b) using a polyclonal antibody for mCAR.

Hepatic microsomes were prepared from frozen liver tissue following a previous method described (Hahn et al., 1989). After comparison of murine Cyp2C sequences, a CYP2C29 specific peptide, IKGFGVVFSNGNRWKEMRRC, was synthesized by Invitrogen (Carlsbad, CA). The specific peptide sequence was coupled to keyhole limpet hemocyanin through the terminal cysteine. Custom polyclonal antibodies specific to CYP2C29 peptide were then produced by Covance Research Products Inc. (Princeton, NJ) as previously described (DeLozier et al., 2004). Hepatic microsomes or partially purified recombinant CYP2C29, CYP2C37, CYP2C38, CYP2C39, CYP2C40, CYP2C44, CYP2C50, CYP2C54, CYP2C55, CYP2C65, CYP2C66, CYP2C70 proteins were electrophoresed under reducing conditions using 10% Bis-Tris NuPAGE precast minigels (Invitrogen) in 1X NuPAGE MOPS SDS running buffer (Invitrogen). Proteins were then transferred to precut nitrocellulose membranes (Invitrogen) in 1X NuPAGE transfer buffer (Invitrogen) supplemented with 10% methanol. Membranes were immunoblotted with rabbit anti-

CYP2C29 peptide-specific antibody (1:500) and donkey anti-rabbit IgG conjugated to horseradish peroxidase from Amersham Biosciences Inc. (Piscataway, NJ). Protein bands were visualized using SuperSignal West Pico Chemiluminescent Substrate from Pierce and Biomax MR Film (Kodak, Rochester, NY).

2.4 Results

Drug Response of CYP2C29 mRNA

In initial experiments, quantitative RT-PCR was used to determine the induction response of CYP2C29 mRNA. Phenytoin and PB increased CYP2C29 mRNA ~5 fold and ~2.5 fold, respectively, in C57BL/6 mice (Fig. 2.1). CYP2B10 mRNA, a positive control, was increased ~ 25 fold by PB. An ~122 fold induction of CYP2B10 mRNA was observed in response to phenytoin. Thus, CYP2C29 and CYP2B10 mRNAs were induced by phenytoin to a greater extent than PB at the concentrations tested.

Hepatic microsomes were prepared from individual mice and subjected to SDS-gel electrophoresis and immunoblot analysis to determine CYP2C29 protein content following phenytoin treatment. The immunoblot showed that primarily a single immunoreactive band ~ 50 kD was detected in all microsome preparations analyzed (Fig. 2.2). Previous Immunoblot analysis was performed to determine the CYP2C29 peptide antibody specificity against different partially purified recombinant CYP2C proteins. These results showed that minimal cross-reactivity occurred with this CYP2C29 peptide antibody preparation (Fig 2.3). Taken together, these results suggest that CYP2C29 protein is induced following phenytoin treatment.

Element Identification and Binding Analysis

A software-based analysis (SeqLab, GCG) of 10 kb of the 5'-flanking region of *Cyp2c29* revealed two DR-4 imperfect repeats of AGGTCA located at -1371 bp upstream of the translation start site which we herein designate as a phenytoin responsive module (PHREM) (Fig. 2.4). These motifs resemble known CAR-binding sites within the enhancer modules of the *Cyp2b10* and *CYP2B6* genes (Honkakoski et al., 1998a; Sueyoshi, 1999; Sueyoshi and Negishi, 2001). Additionally, a putative c/EBP site was discovered separating the DR-4 motifs of *Cyp2c29* in contrast to the NF-1 site found in *Cyp2b10* (Fig. 2.4) (Honkakoski et al., 1998b). Using EMSA, both putative DR-4 motifs were examined for their ability to bind nuclear receptors CAR and PXR. Both NR1 and NR2 sites bound to mCAR in the presence of RXR, which was competed out with 100 fold molar excess of nonradiolabeled oligonucleotides (Fig. 2.5). Human PXR bound weakly to both DR-4 sites of the *Cyp2c29* gene relative to the *Cyp2B6* NR1 control.

In Vitro PHREM Analysis

To evaluate the functional significance of each CAR-binding element in the regulation of the *Cyp2c29* gene, we constructed various luciferase reporter plasmids containing 5' flanking sequence (-1.5 kb) of *Cyp2c29* and transfected them into HepG2 cells. Each of the DR-4 sites, NR1 and NR2, were mutated individually and in combination to evaluate their contribution to the function of the module (Fig 2.6). The *Cyp2c29* wild-type luciferase construct was activated ~75 fold by mCAR in the

absence of exogenous ligand. Mutation of the NR1 or NR2 binding elements significantly reduced mCAR activation in a non-additive manner to ~23% and ~20% of the wild-type response, respectively (Fig. 2.7). The *Cyp2c29* double mutant, NR1/2mut, further reduced mCAR activation to ~12% of the wild-type response. Mutations within the putative c/EBP binding site reduced mCAR activation by ~50%.

Known ligands of mCAR, androstenol and TCPOBOP(Forman et al., 1998; Tzameli et al., 2000), were used to further investigate the ability of mCAR to regulate *Cyp2c29* transcription. Addition of 10 μM androstenol repressed transcriptional activation of the wild-type *Cyp2c29* reporter. This response was derepressed by the addition of 250 nM TCPOBOP (Fig. 2.7). NR1 mutant and NR2 mutant transcriptional activation was marginally repressed by androstenol, but was not consistently derepressed by the addition of TCPOBOP. The mutations of both elements resulted in the complete loss of mCAR ligand responsiveness. Although mutation of the c/EBP site decreased constitutive activation by mCAR, repression by androstenol and derepression by TCPOBOP were similar to that of the wild-type response (Fig. 2.7). In contrast to TCPOBOP, neither phenytoin (100 μM) nor PB (1 mM) was able to reverse androstenol repression of mCAR constitutive activity on the wild-type luciferase construct (Fig. 2.8). Nor did phenytoin or phenobarbital have an effect on promoter activity in the presence or absence of mCAR. The NR1 mutation had a slightly greater effect on mCAR activation than the NR2 or NR1/NR2 mutations, consistent with results in (Fig 2.7).

Nuclear Translocation of CAR

To determine whether phenytoin could induce nuclear translocation of CAR in a manner similar to that observed following PB exposure, immunoblots were performed on hepatic nuclear extracts isolated from mice treated with corn oil, phenytoin (80mg/kg), or PB (80mg/kg) using an antibody to mCAR. Immunoblot analysis showed a marked increase in nuclear CAR content in phenytoin and PB treated mice, indicating that both phenytoin and PB initiate nuclear translocation of CAR (Fig. 2.9).

In vivo PHREM Analysis

When *Cyp2c29* -1.5 kb promoter constructs were injected into C3H mice, luciferase activity of the *Cyp2c29* wild-type reporter was induced ~8 fold by phenytoin and ~3 fold by PB (Fig. 2.10). Mutation of both DR-4 sites (Fig. 2.4) within the *Cyp2c29* NR1/2mut promoter construct eliminated induction of luciferase activity.

C3H Wild-type vs CAR-null

To further investigate the role of mCAR in the induction of *Cyp2c29* by phenytoin and PB, wild-type and CAR-null mice were treated with either inducer. Quantitative RT-PCR indicated that CYP2C29 mRNA was elevated ~6.2 fold in response to phenytoin and ~2.6 fold in response to PB in wild-type mice (Fig. 2.11A), which is consistent with our previous results. Additionally, CYP2B10 mRNA was increased ~332 fold by phenytoin and ~60 fold by PB in wild-type mice. In

CAR-null mice, CYP2B10 mRNA induction by PB was eliminated, while phenytoin induction was ~99.3% lower than the response observed in wild-type mice. Induction of CYP2C29 mRNA by PB and phenytoin in CAR-null mice was decreased by ~96% and ~97% respectively, compared to the response in wild-type mice. Surprisingly, the constitutive level of CYP2C29 mRNA was dramatically reduced in the CAR-null mice (~77 fold) (Fig. 2.11B), while constitutive expression of CYP2B10 mRNA was slightly reduced (~6 fold).

2.5 Discussion

In the present study, we utilized CAR-null mice to show that phenytoin induces CYP2C29 mRNA via the nuclear receptor CAR. Furthermore, we identified a PHREM in the upstream region of the *Cyp2c29* gene, which mediates phenytoin induction. Importantly, we also show CAR is involved in regulating the constitutive expression of the *Cyp2c29* gene.

While phenytoin and PB were both able to induce both CYP2C29 and CYP2B10 mRNA, phenytoin was a stronger inducer of both P450s at the concentrations studied. Immunoblot analysis indicated that phenytoin induced CYP2C29 protein. These results suggested that CYP2C29 induction by phenytoin was governed by a transcriptional mechanism. We identified a novel PHREM located at -1371 bp upstream of the *Cyp2c29* gene, similar to the PBREM found within both *Cyp2b10* and *CYP2B6* genes (Honkakoski et al., 1998a; Sueyoshi, 1999). Mutagenesis of both DR-4 elements of the PHREM abolished induction in phenytoin and PB treated mice consistent with similar studies of the PBREM

removing PB induction of the *Cyp2b10* gene (Honkakoski et al., 1998a; Honkakoski et al., 1998b; Sueyoshi, 1999). Additionally, the results of the *in vivo* reporter assays were consistent with the magnitude of the mRNA induction suggesting that the PHREM is the principle CAR-responsive module within the 5'-flanking region of the *Cyp2c29* gene. We observed an unusual arrangement of the DR-4 elements within the PHREM, as they are inverted in relation to each other. This topological arrangement of DR-4 elements is unlike those found in the PBREM motifs of the *Cyp2b10* or *CYP2B6* genes (Honkakoski et al., 1998a; Honkakoski et al., 1998b; Sueyoshi, 1999), and could be important in regulatory properties such as nuclear receptor specificity. Both elements of the PHREM were capable of binding CAR and to a much lesser extent PXR which is consistent with the ability of CAR and PXR to recognize similar direct hexamer repeats, but suggests marked specificity for CAR (Honkakoski and Negishi, 2000; Sueyoshi and Negishi, 2001; Liddle and Goodwin, 2002; Makinen et al., 2002).

Our studies demonstrate that the PHREM of *Cyp2c29* was markedly activated by mCAR (~75 fold) and that this activation could be modulated using mCAR specific ligands (androstenol and TCPOBOP) (Forman et al., 1998; Tzameli et al., 2000) indicative of CAR regulated genes (Honkakoski et al., 1998b; Honkakoski and Negishi, 2000; Sueyoshi and Negishi, 2001). Mutagenesis of NR1 and NR2 abolished drug responsiveness indicating that both DR-4 elements are necessary for mCAR-mediated induction. Interestingly, mutagenesis of either DR-4 motif reduced mCAR constitutive activation to ~18-23% of the wild-type response suggesting that for full activation, the individual DR-4 elements interact synergistically with mCAR.

Mutagenesis of the putative c/EBP binding site found in the PHREM resulted in reduced mCAR constitutive activation but did not abolish drug responsiveness indicating that the c/EBP binding site may be important for the full constitutive activation of the PHREM by mCAR. These results are somewhat analogous to the results of the NF1 mutations within the PBREM of *Cyp2b10* (Honkakoski et al., 1998b).

In contrast to TCPOBOP, PB is not believed to bind mCAR (Tzameli et al., 2000; Sueyoshi and Negishi, 2001). Instead, induction is thought to occur via a presently unidentified signaling pathway that induces nuclear translocation of the constitutively active CAR. Neither phenytoin nor PB was able to reverse androstenol repression of mCAR in transiently cotransfected HepG2 cells with *Cyp2c29* wild-type reporter. Thus, phenytoin is presumably not a ligand for mCAR. However, immunoblot analysis of hepatic nuclear extracts from control, PB, or phenytoin treated mice indicated that phenytoin induces nuclear translocation of mCAR in a manner similar to PB (Fig. 7), thereby eliciting the induction response of both *Cyp2c29* and *Cyp2b10* genes.

Although phenytoin induction is not completely eliminated in CAR-null mice, the induction of *Cyp2c29* and *Cyp2b10* were reduced to ~3% and ~0.7% that of the wild-type response, respectively. The residual induction may possibly be explained by nuclear receptor cross-talk since CAR and PXR recognize similar binding motifs (Honkakoski and Negishi, 2000; Sueyoshi and Negishi, 2001; Liddle and Goodwin, 2002; Makinen et al., 2002). Therefore, double null (CAR and PXR) mice would be

useful to explore this phenomenon. Alternatively, other non-CAR mediated mechanisms may account for the residual increase in CAR-null mice.

Although CAR is currently perceived as a receptor that is involved primarily in drug induction (Sueyoshi, 1999; Honkakoski and Negishi, 2000; Sueyoshi and Negishi, 2001), recent evidence has suggested that CAR may play a more diverse role in gene regulation. CAR has been shown to be involved in induction, repression, and transcriptional blocking of induction and repression by PB (Ueda et al., 2002). In our study, quantitative RT-PCR in CAR-null mice revealed that constitutive expression of *Cyp2c29* was substantially reduced (~77 fold). These results clearly establish a direct or indirect role for CAR in the constitutive regulation of *Cyp2c29* and are consistent with studies showing that CAR is a multifaceted transcription factor (Ueda et al., 2002).

In summary, our studies have demonstrated that phenytoin can induce both *Cyp2c29* and *Cyp2b10*, potentially providing a useful tool for investigating P450 regulation. Using CAR-null mice, we have demonstrated that CAR is responsible for the induction of *Cyp2b10* and *Cyp2c29* by phenytoin, identifying a new CAR activator. CAR activation was localized to a novel PHREM located at -1371 bp upstream of the *Cyp2c29* translation start site. Finally, CAR was shown to be involved in the constitutive expression of *Cyp2c29* as suggested by the reduction of the basal CYP2C29 mRNA content in the CAR-null mice providing more evidence to support the hypothesis that CAR has multiple roles in gene regulation.

2.6 References Cited

DeLozier TC, Tsao CC, Coulter SJ, Foley J, Bradbury JA, Zeldin DC and Goldstein JA (2004) CYP2C44, a new murine CYP2C that metabolizes arachidonic acid to unique stereospecific products. *J Pharmacol Exp Ther* 310:845-854.

Ferguson SS, LeCluyse EL, Negishi M and Goldstein JA (2002) Regulation of human CYP2C9 by the constitutive androstane receptor: discovery of a new distal binding site. *Mol Pharmacol* 62:737-746.

Forman BM, Tzameli I, Choi HS, Chen J, Simha D, Seol W, Evans RM and Moore DD (1998) Androstane metabolites bind to and deactivate the nuclear receptor CAR-beta. *Nature* 395:612-615.

Gerbal-Chaloin S, Daujat M, Pascussi JM, Pichard-Garcia L, Vilarem MJ and Maurel P (2002) Transcriptional regulation of CYP2C9 gene. Role of glucocorticoid receptor and constitutive androstane receptor. *J Biol Chem* 277:209-217.

Goodwin B, Hodgson, E., D"Costa, D.J., Robertson, G.R., Liddle, C. (2002) Transcriptional Regulation of the Human *CYP3A4* Gene by the Constitutive Androstane Receptor. *Molecular Pharmacology* 62:359-365.

Hahn ME, Goldstein JA, Linko P and Gasiewicz TA (1989) Interaction of hexachlorobenzene with the receptor for 2,3,7,8-tetrachlorodibenzo-p-dioxin in vitro and in vivo. Evidence that hexachlorobenzene is a weak Ah receptor agonist. *Arch Biochem Biophys* 270:344-355.

Honkakoski P, Moore R, Washburn KA and Negishi M (1998a) Activation by diverse xenochemicals of the 51-base pair phenobarbital- responsive enhancer module in the CYP2B10 gene. *Mol Pharmacol* 53:597-601.

Honkakoski P and Negishi M (2000) Regulation of cytochrome P450 (CYP) genes by nuclear receptors. *Biochem J* 347:321-337.

Honkakoski P, Zelko I, Sueyoshi T and Negishi M (1998b) The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the CYP2B gene. *Mol Cell Biol* 18:5652-5658.

Kast HR, Goodwin B, Tarr PT, Jones SA, Anisfeld AM, Stoltz CM, Tontonoz P, Kliewer S, Willson TM and Edwards PA (2002) Regulation of multidrug resistance-associated protein 2 (ABCC2) by the nuclear receptors pregnane X receptor, farnesoid X-activated receptor, and constitutive androstane receptor. *J Biol Chem* 277:2908-2915.

Kliewer SA, Goodwin B and Willson TM (2002) The nuclear pregnane X receptor: a key regulator of xenobiotic metabolism. *Endocr Rev* 23:687-702.

Kobayashi K, Sueyoshi T, Inoue K, Moore R and Negishi M (2003) Cytoplasmic accumulation of the nuclear receptor CAR by a tetratricopeptide repeat protein in HepG2 cells. *Mol Pharmacol* 64:1069-1075.

Liddle C and Goodwin B (2002) Regulation of hepatic drug metabolism: role of the nuclear receptors PXR and CAR. *Semin Liver Dis* 22:115-122.

Luo G, Cunningham M, Kim S, Burn T, Lin J, Sinz M, Hamilton G, Rizzo C, Jolley S, Gilbert D, Downey A, Mudra D, Graham R, Carroll K, Xie J, Madan A, Parkinson A, Christ D, Selling B, LeCluyse E and Gan LS (2002) CYP3A4 induction by drugs: correlation between a pregnane X receptor reporter gene assay and CYP3A4 expression in human hepatocytes. *Drug Metab Dispos* 30:795-804.

Luo G, Zeldin DC, Blaisdell JA, Hodgson E and Goldstein JA (1998) Cloning and expression of murine CYP2Cs and their ability to metabolize arachidonic acid. *Archives of Biochemistry and Biophysics* 357:45-57.

Makinen J, Frank C, Jyrkkarinne J, Gynther J, Carlberg C and Honkakoski P (2002) Modulation of mouse and human phenobarbital-responsive enhancer module by nuclear receptors. *Mol Pharmacol* 62:366-378.

Meyer RP, Hagemeyer CE, Knoth R, Kurz G and Volk B (2001a) Oxidative hydrolysis of scoparone by cytochrome p450 CYP2C29 reveals a novel metabolite. *Biochem Biophys Res Commun* 285:32-39.

Meyer RP, Knoth R, Schiltz E and Volk B (2001b) Possible function of astrocyte cytochrome P450 in control of xenobiotic phenytoin in the brain: in vitro studies on murine astrocyte primary cultures. *Exp Neurol* 167:376-384.

Raucy JL (2003) Regulation of CYP3A4 expression in human hepatocytes by pharmaceuticals and natural products. *Drug Metab Dispos* 31:533-539.

Sueyoshi T, Kawamoto, T., Zelko, I., Honkakoski, P. and Negishi, M. (1999) The repressed nuclear receptor CAR responds to phenobarbital in activating the human CYP2B6 gene. *Journal of Biological Chemistry* 274:6043-6046.

Sueyoshi T, Kobayashi R, Nishio K, Aida K, Moore R, Wada T, Handa H and Negishi M (1995) A nuclear factor (NF2d9) that binds to the male-specific P450 (Cyp 2d-9) gene in mouse liver. *Mol Cell Biol* 15:4158-4166.

Sueyoshi T and Negishi M (2001) Phenobarbital response elements of cytochrome P450 genes and nuclear receptors. *Annu Rev Pharmacol Toxicol* 41:123-143.

Tzameli I, Pissios P, Schuetz EG and Moore DD (2000) The xenobiotic compound 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene is an agonist ligand for the nuclear receptor CAR. *Mol Cell Biol* 20:2951-2958.

Ueda A, Hamadeh HK, Webb HK, Yamamoto Y, Sueyoshi T, Afshari CA, Lehmann JM and Negishi M (2002) Diverse roles of the nuclear orphan receptor CAR in regulating hepatic genes in response to phenobarbital. *Mol Pharmacol* 61:1-6.

Wang H, Faucette SR, Gilbert D, Jolley SL, Sueyoshi T, Negishi M and LeCluyse EL (2003) Glucocorticoid receptor enhancement of pregnane X receptor-mediated CYP2B6 regulation in primary human hepatocytes. *Drug Metab Dispos* 31:620-630.

Yoshinari K, Kobayashi K, Moore R, Kawamoto T and Negishi M (2003) Identification of the nuclear receptor CAR:HSP90 complex in mouse liver and recruitment of protein phosphatase 2A in response to phenobarbital. *FEBS Lett* 548:17-20.

TABLE 2.1. Cloning, Sequencing, and Quantitative PCR Oligonucleotides

#	Oligonucleotides	Oligonucleotide Sequence (5'→3')
	Cloning Primers	
52	Cyp2c29-1.5kb	CTCGAGGATATCTGAGATTGATTCATAC
53	Cyp2c29-1.5kb	CCATGGTAGCGAACCTCCTTACTG
	Sequencing Primers	
54	Cyp2c29_SQ-1017F	AGTGGAAAGTGGAGAATTTTAAGA
55	Cyp2c29_SQ-539F	ATTTCTAGCTATTGGGGCAAT
72	Cyp2c29_SQ-200F	TCAAATGGGCACTCTGTAAAG
	Quantitative PCR Primers	
56	Cyp2c29_RT@220CDF	GCTCAAAGCCTACTGTCA
57	Cyp2c29_RT@390CDR	CATGAGTGTAAATCGTCTCA
43	β-ActinF	TCATGAAGTGTGACGTTGACATCCGT
44	β-ActinR	CCTAGAAGCATTTGCGGTGCACGATG
80	Cyp2b10_RT@506CDF	ACCCCACGTTCCTCTTCCA
81	Cyp2b10_RT@606CDR	CAGCAGGCGCAAGAACTGA

TABLE 2.2. EMSA and Mutagenesis Oligonulceotides. Mutated nucleotides in bold

#	Oligonucleotides	Oligonucleotide Sequence (5'→3')
	EMSA Oligos	
48	Cyp2c29-1370F	CTAGTTGGGTCACTGAAGTTCTGA
53	Cyp2c29-1370R	CTAGTCAGAACTTCAGTGACCCAA
50	Cyp2c29-1337F	CTAGAGTAAACTTTTTTGACTTCT
51	Cyp2c29-1337R	CTAGAGAAGTCAAAAAAGTTTACT
	CYP2B6-NR1F	CTAGACTGTACTTTCCTGACCCTG
	CYP2B6-NR1R	CTAGCAGGGTCAGGAAAGTACAGT
	Mutagenesis Primers	
58	MUT_NR1_2c29F	GATAACTGTGACATTGGG**GGG**CTGAAGTTCTGAACAT
60	MUT_NR1_2c29R	ATGTTCAGAACTTCAG**CCC**CCCAATGTCACAGTTATC
59	MUT_NR2_2c29F	CATTACAAAACAAGTAA**TAA**TTTTTGACTTCTAAGTTAA
61	MUT_NR2_2c29R	TTAACTTAGAAGTCAAAAA**TTA**TTACTTGTTTTGTAATG
64	c/EBPmut_2c29F	TCACTGAAGTTCTGAACA**CC**A**T**AA**G**ACAAGTAAACTTTTTTGACT
65	c/EBPmut_2c29R	AGTCAAAAAAGTTTACTTGT**C**TT**A**T**GG**TGTTCAGAACTTCAGTGA

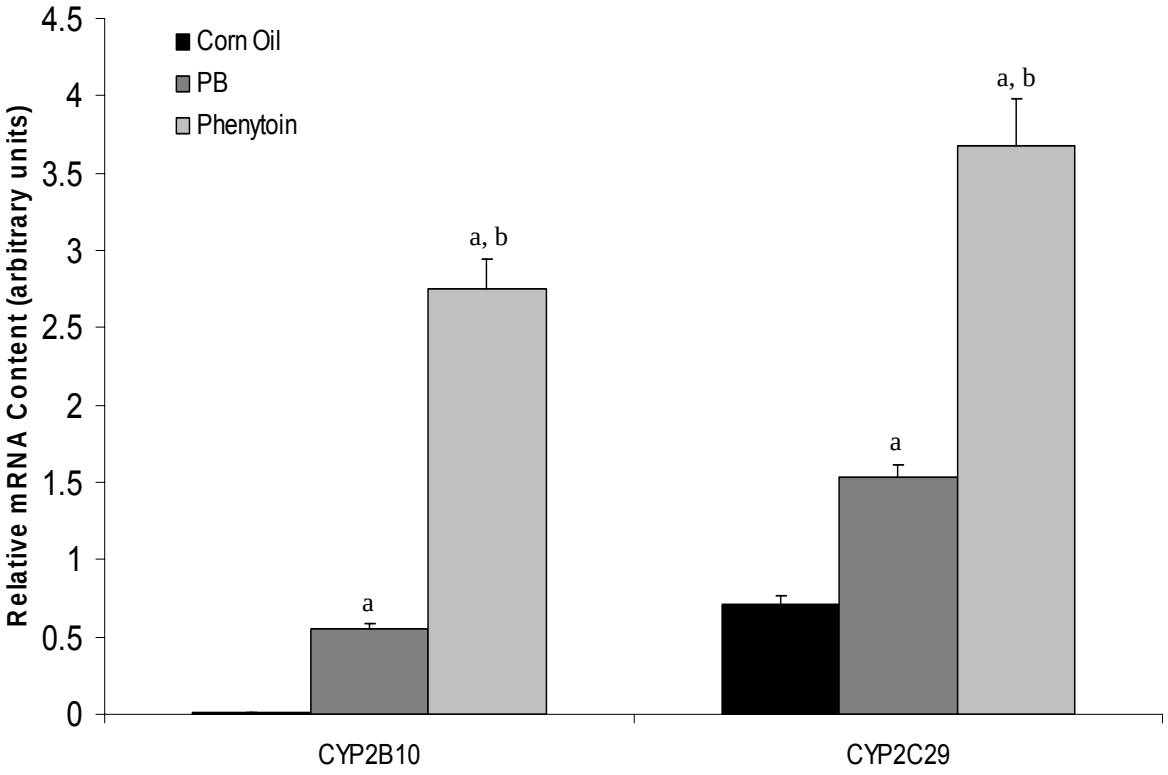

Figure 2.1- Drug response of hepatic CYP2C29 and CYP2B10 mRNA in C57BL/6 mice. Mice were treated with phenytoin (80 mg/kg), PB (80 mg/kg), or corn oil (10 ml/kg) via oral gavage for 4 days. Mice were sacrificed 24 hrs after the fourth dose and livers were removed for total RNA isolation. Quantitative RT-PCR was performed to determine the CYP2C29 and CYP2B10 mRNA content. Target gene values are normalized to β-Actin values. CYP2B10 mRNA was increased ~122 fold and ~34 fold by phenytoin and PB, respectively. Similarly, CYP2C29 mRNA content increased ~5.2 fold with phenytoin and ~2.2 fold with PB. RT-PCR products were analyzed by gel electrophoresis, dissociation curve analyses, and autosequencing to verify gene product specificity. Values expressed above represent relative amounts of gene specific mRNA $\pm$ SE. [a]Significantly greater than corn oil treated mice, $p < 0.05$. [b]Significantly greater than PB treated mice, $p \leq 0.05$.

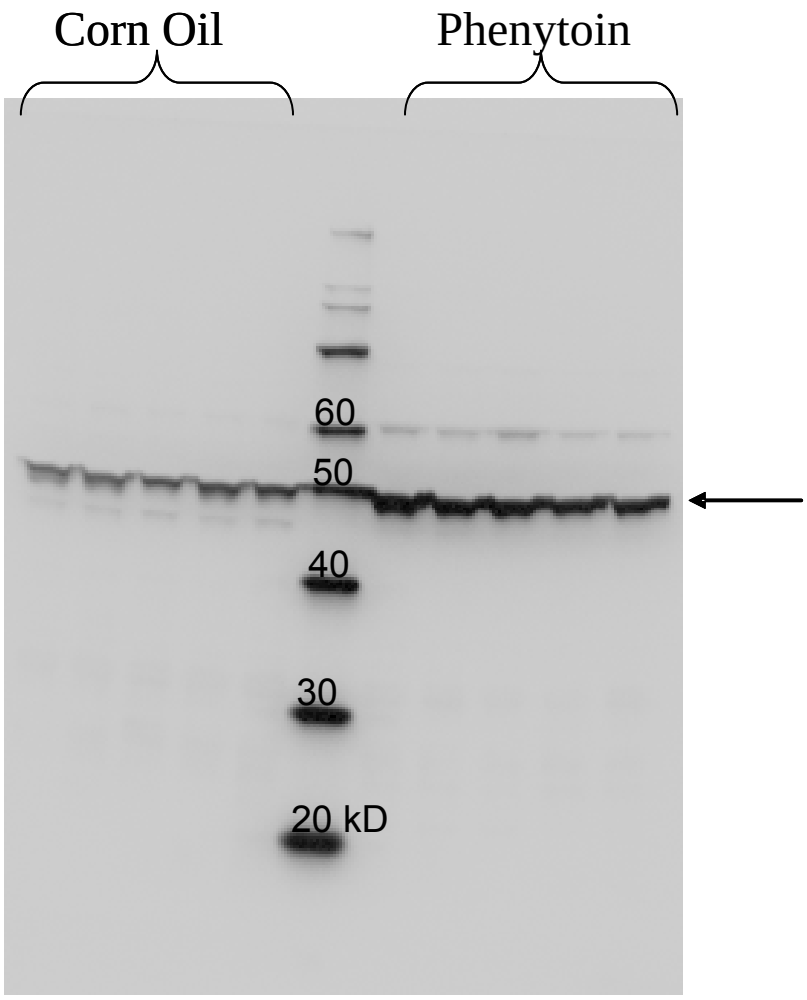

Figure 2.2- Drug response of hepatic CYP2C29 protein in C3H wild-type mice. Mice were treated with vehicle (corn oil) or phenytoin (80 mg/kg) for four consecutive days as above. The livers were removed for hepatic microsome isolation. Individual hepatic microsome preparations (30 µg of total protein) were analyzed on a 10% Bis/Tris NuPAGE gel (Invitrogen) and then transferred to a nitrocellulose membrane. Arrow denotes CYP2C29 immunoreactive bands.

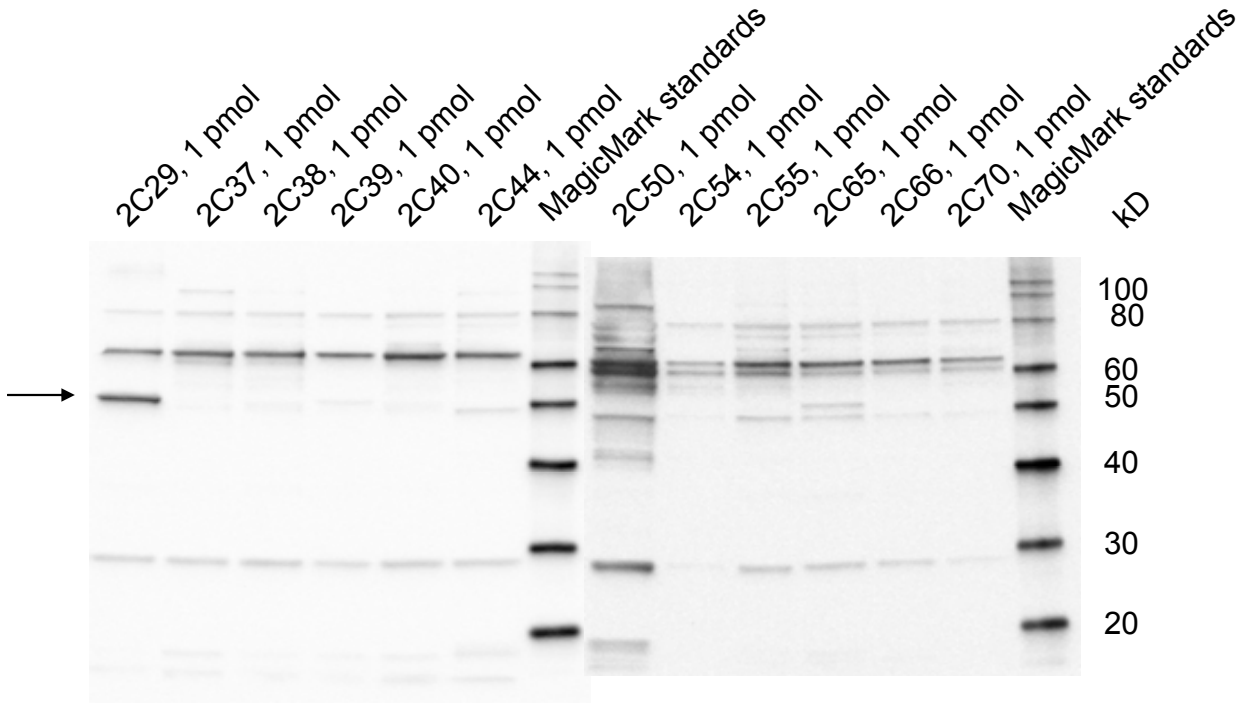

Figure 2.3- Specificity of the CYP2C29 peptide antibody. 1 pmol of partially purified recombinant protein for each CYP2C isoform identified above was analyzed on a 10% Bis/Tris NuPAGE gel (Invitrogen) and then transferred to a nitrocellulose membrane. Strong immunoreactive band in CYP2C29 lane at ~50 kD was observed, denoted by arrow. Minimal cross-reactivity was observed in the other lanes at ~50 kD.

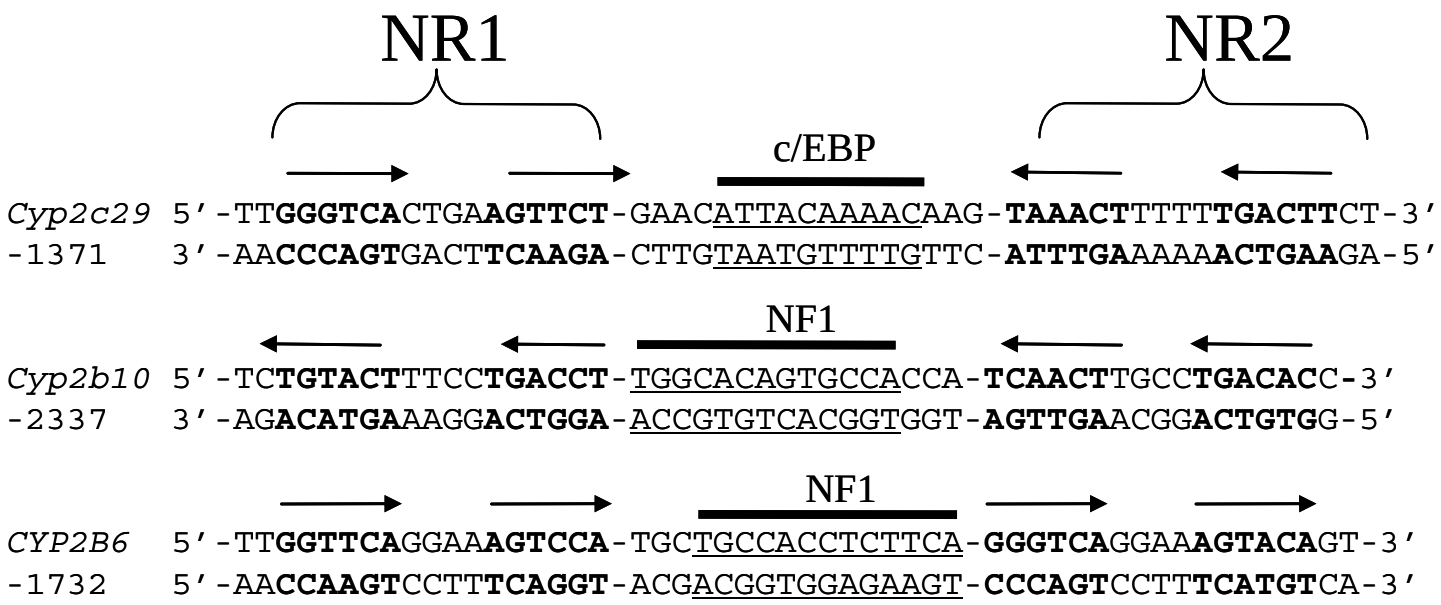

Figure 2.4- PBREM & PHREM topology comparison. A schematic comparing the topology of the PHREM in the upstream sequence of *Cyp*2c29 compared to the PBREMs identified in the upstream sequences of murine *Cyp*2b10 and human *CYP*2B6. Sequences of each DR-4 element are shown in bold for all three genes, arrows denote the orientation of elements, and NF-1 and c/EBP sites are underlined.

	2B6NR1						2c29NR1						2c29NR2					
mCAR/hRXR	x	x	x				x	x	x				x	x	x			
hPXR/hRXR				x	x	x				x	x	x				x	x	x
10x Cold Competitor		x			x			x			x			x			x	
100x Cold Competitor			x			x			x			x			x			x

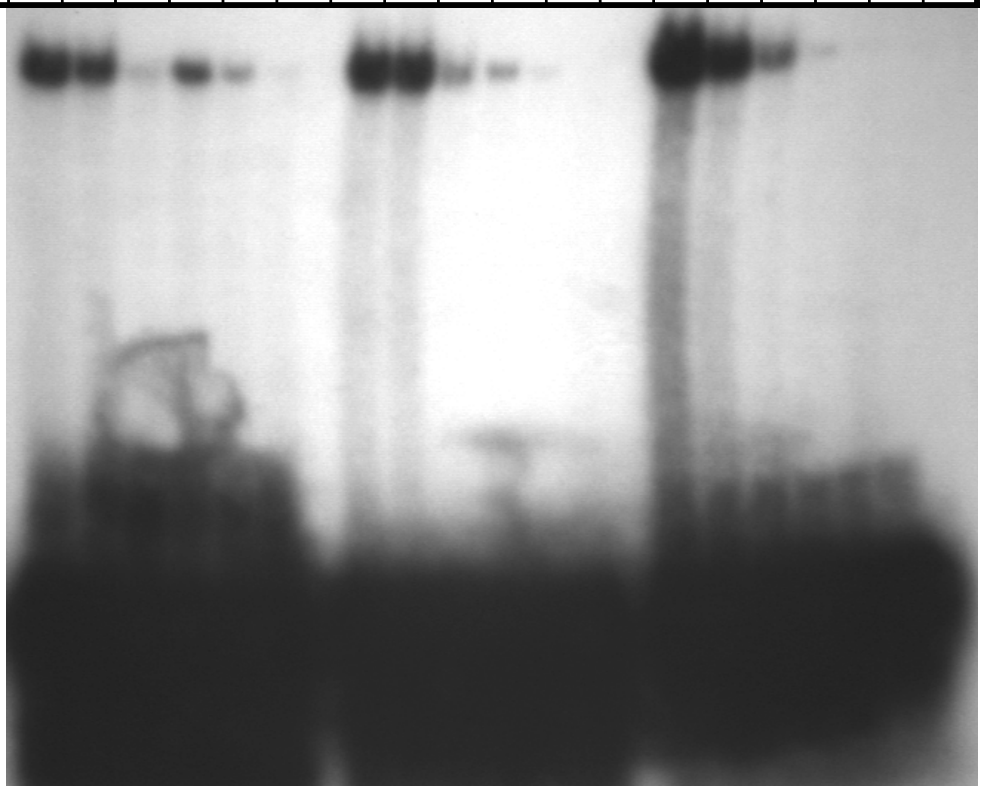

Figure 2.5- Analysis of NR1 and NR2 binding to CAR and PXR by electrophoretic mobility shift assay. Radio-labeled NR1 and NR2 (35,000 cpm ^{32}P) oligonucleotides were incubated with *in vitro* transcribed and translated mCAR/RXR or hPXR/RXR proteins. In parallel experiments, incubation was performed in the presence of 10 and 100 fold molar excess of nonradiolabeled NR1 or NR2 oligonucleotides.

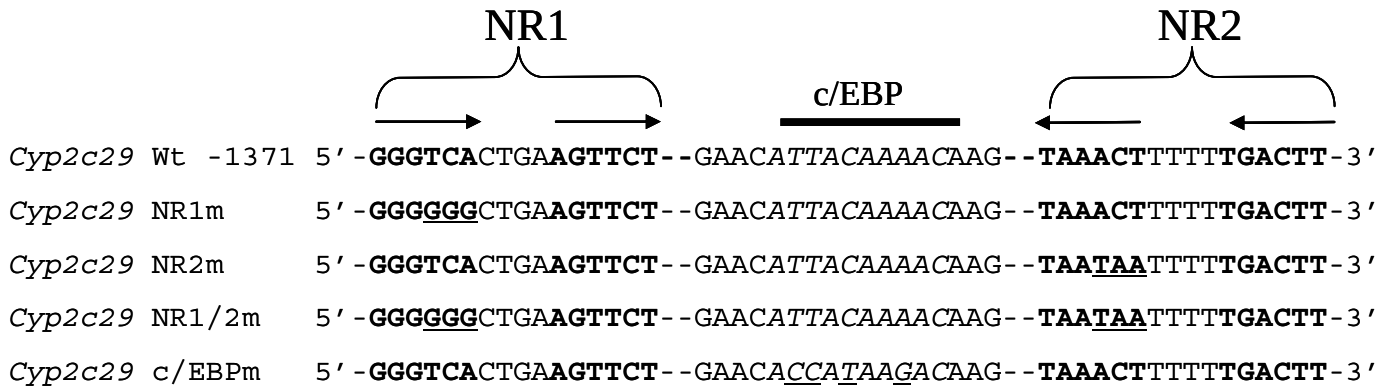

Figure 2.6- Wild-type and mutant PHREM sequences in -1.5 kb luciferase reporter constructs. Mutated nucleotides are underlined.

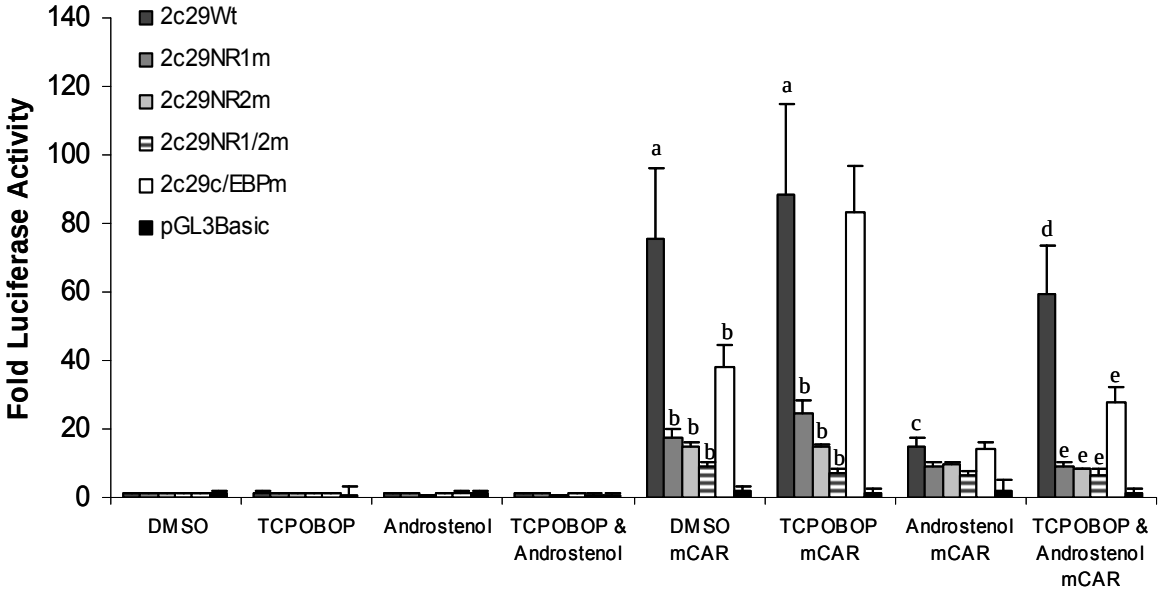

Figure 2.7- Functional analysis of the proximal DR-4 elements in the 5'-flanking region of *Cyp*2c29. Transfection experiments of the *Cyp*2c29 promoter constructs to evaluate the effects of mCAR and its ligands on gene reporter activity. Luciferase reporters containing wild-type and mutant PHREM of the *Cyp*2c29 gene were transfected in HepG2 cells with mCAR expression vector (pCR3.0-mCAR) and pRL-TK for internal transfection control. Modulation of the constitutive activity of mCAR was accomplished by treatment with androstenol (10 μM) or TCPOBOP (250 nM) / androstenol (10 μM) for 24hrs. Reporter activation is expressed as a ratio of normalized luciferase activity in the presence of mCAR to the activity in the absence of mCAR ± SE. [a]Cotransfection of mCAR significantly increased activity of the *Cyp2c29* Wt promoter in cells treated with DMSO or TCPOBOP, $p \leq 0.05$, [b]Activity of the *Cyp2c29* mutant constructs was significantly lower than that of *Cyp2c29* Wt constructs in cells cotransfected with mCAR and treated with DMSO, or TCPOBOP, $p \leq 0.05$ [c]Androstenol significantly repressed activity of the *Cyp2c29* Wt promoter construct in cells cotransfected with mCAR, $p \leq 0.05$ [d]TCPOBOP derepressed activity of the *Cyp2c29* Wt promoter in cells cotransfected with mCAR and treated with androstenol , $p \leq 0.05$ [e]Activity of *Cyp2c29* mutant constructs was significantly lower than that of *Cyp2c29* Wt in cells cotransfected with mCAR and treated with androstenol and TCPOBOP, $p \leq 0.05$.

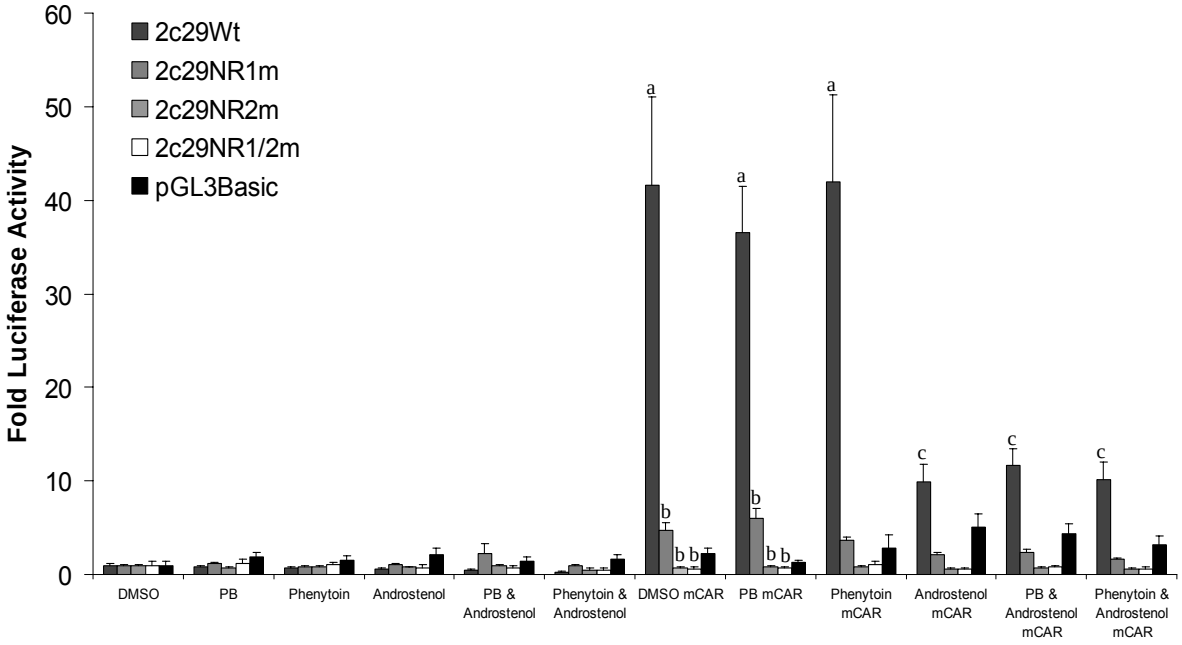

Figure 2.8- Phenytoin and PB are unable to reverse androstenol repression of mCAR constitutive activity in HepG2 transient transfections of *Cyp2c29* promoter constructs. Luciferase reporters containing wild-type and mutant PHREM of the *Cyp2c29* gene were transfected in HepG2 cells with mCAR expression vector (pCR3.0-mCAR) and pRL-TK for internal transfection control. Reporter activation is expressed as a ratio of normalized luciferase activity in the presence of mCAR to the activity in the absence of mCAR $\pm$ SE. [a]Cotransfection of mCAR significantly increased activity of *Cyp2c29* Wt promoter in cells treated with DMSO, PB (1 mM), or phenytoin (100 µM), $p \leq 0.05$, [b]Activity of the *Cyp2c29* mutant constructs was significantly lower than that of *Cyp2c29* Wt constructs in cells contransfected with mCAR in the presence of DMSO, PB, or phenytoin, $p \leq 0.05$ [c]Androstenol (10 µM) significantly repressed activity of the *Cyp2c29* promoter constructs in cells cotransfected with mCAR, $p \leq 0.05$, regardless of the presence of PB (1 mM) or phenytoin (100 µM).

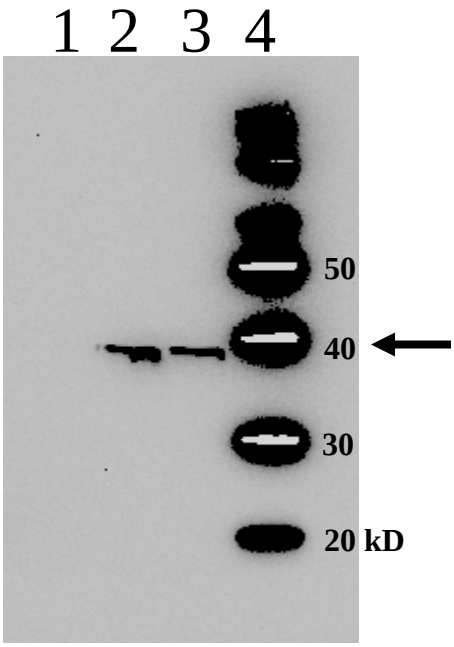

Figure 2.9- Nuclear translocation of mCAR by phenytoin and PB. Hepatic nuclear extracts were isolated from mice 3 hours after treatment with phenytoin (80mg/kg), PB (80mg/kg), or corn oil (10ml/kg) i.p. and subjected to Western blot analysis using an antibody to mCAR. Lane 1 – DMSO, Lane 2 – PB, Lane 3 – phenytoin.

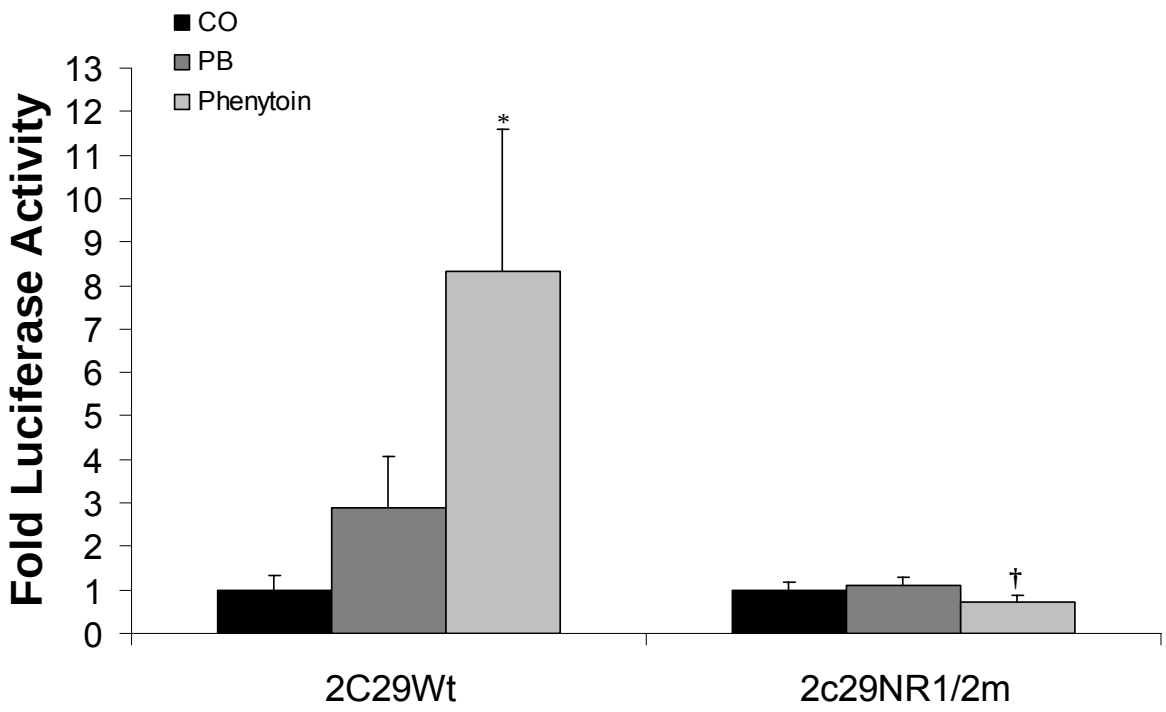

Figure 2.10- *In vivo* identification of a phenytoin responsive enhancer module (PHREM) in the 5'-flanking region of *Cyp*2c29. C3H mice were treated with phenytoin (80 mg/kg), PB (80 mg/kg), or corn oil (10 ml/kg) for 4 days. On the fourth day, wild-type and NR1/2m *Cyp2c29* luciferase promoter constructs and pRL-TK (transfection control plasmid) were injected into mice via tail veins using a gene delivery system from Mirus as described under "Materials and Methods". Animals were sacrificed 24 hrs after tail vein injection, and livers were removed for luciferase assay. Induction is expressed as a ratio of normalized luciferase activity in the presence of drug to the activity in the absence of drug $\pm$ SE. *Significantly greater than corn oil treated mice injected with *Cyp2c29* Wt reporter, $p \leq 0.05$. †Significantly lower than phenytoin treated mice injected with *Cyp2c29* Wt reporter, $p < 0.01$.

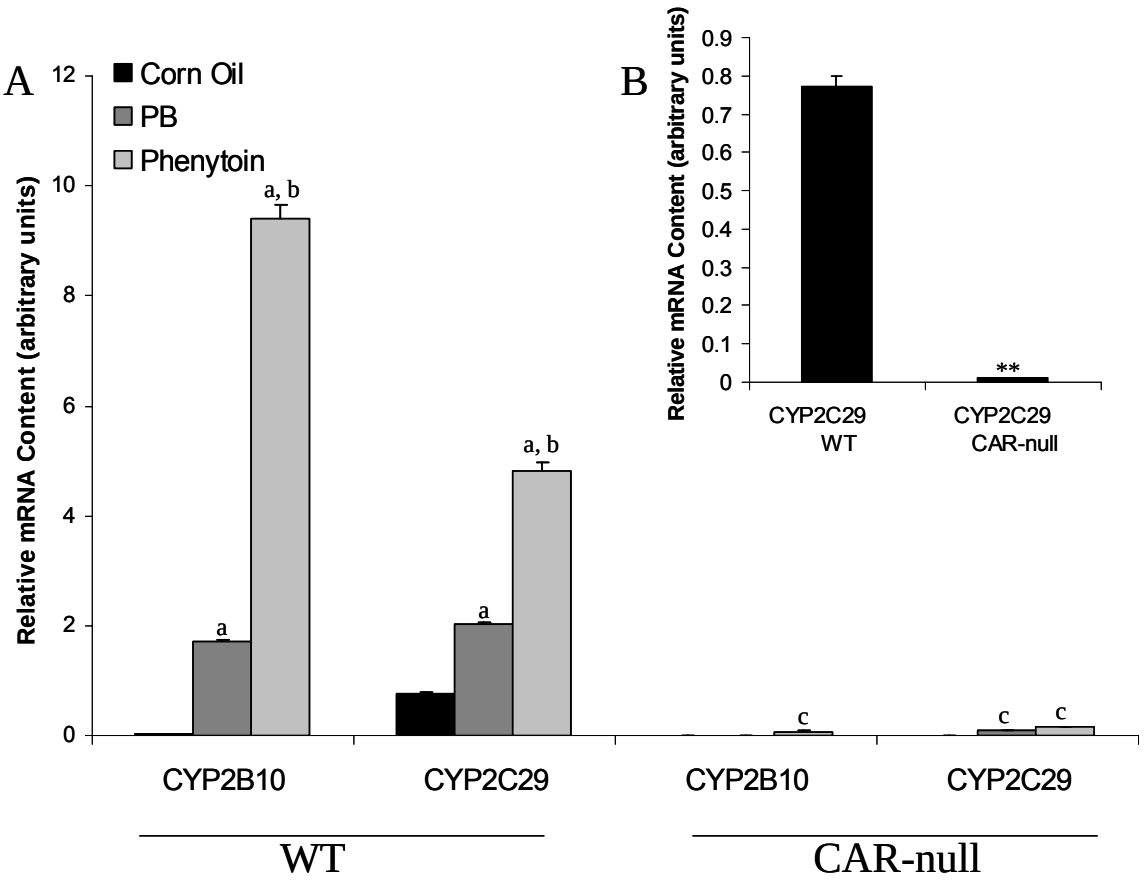

Figure 2.11- Drug response of hepatic CYP2C29 mRNA in C3H wild-type vs CAR-null-mice. A, mice were treated with phenytoin (80 mg/kg), PB (80 mg/kg), or corn oil (10 ml/kg) as previously described under "Experimental Procedures." Mice were sacrificed 24 hrs after the fourth dose and livers were removed for total RNA isolation. Quantitative RT-PCR was performed to determine the CYP2C29 and CYP2B10 mRNA content. Gene specific data were normalized to □-Actin. Values expressed above represent relative amounts of gene specific mRNA ± SE. [a]Significantly greater than corn oil treated wild-type mice, p< 0.01. [b]Significantly greater than PB treated wild-type mice, $p < 0.01$. [c]Drug treated CAR-null mice significantly lower than respective drug treated wild-type mice, $p < 0.01$. B, Wild-type vs CAR-null constitutive expression of *Cyp2c29*. CAR-null mice show a substantial reduction (~77 fold) in *Cyp2c29* constitutive expression indicating that CAR is an important factor in the constitutive regulation of this gene. [**]Significantly lower than corn oil treated wild-type mice, $p < 0.01$.

3. Phenytoin Induction of the *Cyp2c37* Gene is Mediated by the Constitutive Active/Androstane Receptor.

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This manuscript was submitted for publication in May 2006 to the Journal of Drug Metabolism and Disposition.

3.1 Abstract

The CYP2C subfamily of cytochrome P450 monoxygenases is responsible for the metabolism of approximately 20% of therapeutic drugs and many endogenous compounds. These enzymes can be induced by prior treatment with drugs, resulting in changes in drug efficacy. Induction of several CYP2C enzymes by xenobiotics occurs at the transcriptional level and is reported to involve the constitutive androstane receptor (CAR) and/or the pregnane X receptor (PXR). Recently, we reported that the anticonvulsant phenytoin induced both murine *Cyp2c29 and Cyp2b10* and that induction was mediated principally by the nuclear receptor CAR. In the present study, we report the discovery of a new phenytoin inducible murine CYP2C isoform, *Cyp2c37*. Quantitative RT-PCR and immunoblots demonstrate that hepatic CYP2C37 mRNA and protein are induced by phenytoin. We have identified a functional CAR response element (CAR-RE) located at -2791 bp from the translation start site of the *Cyp2c37* gene. Mutation of this CAR-RE in *Cyp2c37* luciferase promoter constructs abolished mouse CAR (mCAR) transactivation in HepG2 cells. Induction of CYP2C37 mRNA by phenytoin was almost completely abolished (~92%) in CAR-null mice. However, phenytoin also induced CYP3A11 mRNA (known to be induced by CAR and PXR agonists), and this induction was only partially decreased in either CAR-null or PXR-null mice. Thus, induction of the *Cyp2c37* gene by phenytoin, like that of the *Cyp2b10* and *Cyp2c29* genes, is regulated primarily by mCAR; however, phenytoin appears to act as an agonist of both mCAR and mouse PXR (mPXR), depending on the promoter context.

3.2 Introduction

The CYP2C subfamily of cytochrome P450 monooxygenases (P450) are responsible for the metabolism of approximately 20% of all clinical drugs in humans as well as many endogenous compounds and other xenobioitics. Examples of clinically important substances metabolized by the human CYP2C enzymes are the anticonvulsant phenytoin, the anticoagulant warfarin, antidiabetic drugs such as tolbutamide, diuretics such as torsemide, and numerous nonsteroidal anti-inflammatory drugs (Goldstein and Blaisdell, 1996; Chen et al., 2003). Recent work has shown that prior exposure of humans to various clinical drugs and herbal medicines can induce the expression of drug-metabolizing enzymes potentially leading to decreases in therapeutic effectiveness or drug-drug interactions (Honkakoski and Negishi, 2000; Gerbal-Chaloin et al., 2001; LeCluyse, 2001; Sueyoshi and Negishi, 2001; Raucy et al., 2002; Pascussi et al., 2003; Pascussi et al., 2004). Human CYP2C8, CYP2C9, and CYP2C19, as well as murine CYP2C29 have been reported to be inducible by xenobiotics (Gerbal-Chaloin et al., 2001; Meyer et al., 2001; Ferguson et al., 2002; Gerbal-Chaloin et al., 2002; Raucy et al., 2002; Chen et al., 2003; Chen et al., 2004; Jackson et al., 2004; Ferguson et al., 2005).

Induction of clinically important P450 enzymes is frequently regulated at the transcriptional level and is mediated by the nuclear receptors such as constitutive androstane receptor (CAR), the pregnane X receptor (PXR), the glucocorticoid receptor (GR), and the perioxisome proliferator-activated receptor (PPAR) via response elements located within the 5'-flanking regions of these genes (Goodwin et

al., 1999; Ferguson et al., 2002; Gerbal-Chaloin et al., 2002; Goodwin, 2002; Goodwin et al., 2002; Johnson et al., 2002; Chen et al., 2003; Pascussi et al., 2003; Chen et al., 2004; Pascussi et al., 2004; Wang et al., 2004; Ferguson et al., 2005). The induction of the human CYP2C isoforms by clinically prescribed drugs such as phenobarbital, rifampicin, and phenytoin has recently been attributed to the nuclear receptors CAR and PXR (Ferguson et al., 2002; Gerbal-Chaloin et al., 2002; Chen et al., 2003; Chen et al., 2004; Ferguson et al., 2005).

The prototypical inducer phenobarbital represents a diverse collection of chemicals that induces the CYP2B and CYP2C enzymes. The induction of the murine *Cyp2b10* and *Cyp2c29* genes by phenobarbital has been shown to be regulated by the nuclear receptor CAR (Honkakoski et al., 1998a; Honkakoski et al., 1998b; Sueyoshi et al., 1999; Honkakoski and Negishi, 2000). Similarly, induction of human *CYP2B6* and murine *Cyp2b10*, and *Cyp2c29* by the anticonvulsant phenytoin has been shown to be mediated primarily by CAR (Wang et al., 2003; Jackson et al., 2004). Upon drug treatment, CAR translocates to the nucleus and heterodimerizes with retinoid X receptor (RXR) before binding to phenobarbital-responsive enhancer module (PBREM) sequences found in the mouse *Cyp2b10* and human *CYP2B6* genes (Honkakoski et al., 1998b; Kawamoto et al., 1999; Sueyoshi et al., 1999; Sueyoshi and Negishi, 2001; Makinen et al., 2002).

Rifampicin, a human PXR agonist, has been shown to induce the human *CYP2B6*, *CYP2C8*, *CYP2C9*, and *CYP3A4* genes via the nuclear receptor PXR (Goodwin et al., 1999; Xie et al., 2000; Staudinger et al., 2001a; Staudinger et al., 2001b; Goodwin et al., 2002). Similarly, the inducer 5-pregnen-3β-ol-20-one-16α-

carbonitrile (PCN) is a specific mPXR ligand and induces the murine gene *Cyp3a11 (Xie et al., 2000; Staudinger et al., 2001a; Staudinger et al., 2001b; Goodwin et al., 2002; Squires et al., 2004)*. Unlike CAR, PXR activation is ligand-dependent, initiating the accumulation of PXR in the nucleus (Squires et al., 2004). Subsequently, PXR heterodimerizes with RXR before binding to xenobiotic-responsive enhancer module (XREM) such as that found in the *CYP3A4* gene (Goodwin et al., 1999; Honkakoski and Negishi, 2000; Goodwin et al., 2002).

There is significant overlap in the activation of the CYP2B, CYP2C, and CYP3A genes by CAR and PXR. Presumably much of this overlap is due to CAR and PXR sharing similar DNA binding preferences (Honkakoski and Negishi, 2000; Makinen et al., 2002; Pascussi et al., 2003; Pascussi et al., 2004). CAR and PXR also share some common activators such as dieldrin, clotrimazole, and phenobarbital with the caveat that species differences are observed (Moore et al., 2000; Wei et al., 2002; Pascussi et al., 2004; Zhang et al., 2004). These circumstances allow for frequent cross-talk between these signaling pathways.

Although significant progress has been made in the transcriptional regulation of the human CYP2C genes, little is known of the murine genes. The murine model provides an excellent system to investigate the mechanisms governing drug induced CYP2C transcription due to the availability of nuclear receptor null-mice. Previously, CYP2C29 was the first murine CYP2C enzyme known to be inducible by phenytoin and phenobarbital (Meyer et al., 2001; Jackson et al., 2004). We reported that phenytoin induction of the murine *Cyp2c29* gene via the phenytoin responsive module (PHREM) was mediated primarily by the nuclear receptor CAR (Jackson et

al., 2004). In the present work we report that a second murine CYP2C gene, *Cyp2c37*, is inducible by phenobarbital and phenytoin. We discover a functional nuclear receptor response element using mutant and deletion *Cyp2c37* luciferase promoter constructs in HepG2 cells. We also determine which nuclear receptor mediates the phenobarbital and phenytoin induction of the *Cyp2c37* gene using CAR-null and PXR-null mice.

3.3 Materials and methods

Materials and Reagents

Phenobarbital (sodium salt), phenytoin (5,5-diphenylhydantoin, sodium salt), 5α-androstenol, 5-pregnen-3β-ol-20-one-16α-carbonitrile (PCN), and 1, 4-bis-[2-(3, 5,-dichloropyridyloxy)] benzene (TCPOBOP) were purchased from Sigma-Aldrich (St. Louis, MO). DMSO and all other common reagents not listed were purchased from Sigma-Aldrich or other common vendors. Cell culture media, fetal bovine serum, 100X Penicillin-streptomycin-glutamine solution and trypsin/EDTA were purchased from Invitrogen (Carlsbad, CA). All oligonucleotides were purchased from Sigma-Genosys (The Woodlands, TX) at 50 nmol scale and desalted. HepG2 cells were purchased from the American Type Culture Collection (Manassas, VA).

Animals

C3H/HeNCrlBR(C3H) mice were purchased from Charles Rivers Laboratory (Wilmington, MA). CAR-null, PXR-null and congenic wild-type mice were obtained from author M.N.. Mice were fed with a standard solid diet and tap water ad libitum for 5 days. Eight to fifteen week old male mice received corn oil (vehicle), phenobarbital (80 mg/kg), or phenytoin (80 mg/kg) once daily via oral gavage at a volume of 10 ml/kg for 4 consecutive days. Mice treated with PCN (80 mg/kg) were orally dosed once daily for 3 consecutive days using corn oil as vehicle. Animals were sacrificed 24 hrs after the last dose. The livers were removed for total RNA isolation and microsome isolation. The NIEHS Animal Care and Use Committee approved all animal procedures.

Total RNA Isolation & Quantitative RT-PCR

Total RNA was extracted using an ABI 6100 Nucleic Acid PrepStation. All chemistry for the ABI 6100 was purchased from Applied Biosystems (Foster City, CA). Total RNA from individual mice was isolated and stored at -80^{o}C. Prior to reverse transcription, equal amounts of RNA from each individual RNA sample was pooled within each experimental group consisting of 3-5 mice according to treatment and genotype. Quantitative RT-PCR analysis was performed using a two step process. An initial reaction with MuLV Reverse Transcriptase (Applied Biosystems) followed by a subsequent quantitative PCR reaction using 2X SYBR Green Master Mix (Applied Biosystems). Reverse transcription was performed with 100 ng of total RNA combined with 1X PCR Buffer II, 0.4 µl (8 units) of Rnase inhibitor (Applied Biosystems), 5.5 mM $MgCl_2$, 0.5 mM dATP, dCTP, dTTP, and dGTP (each), 2.5 µM random hexamers (Applied Biosystems), and 0.5 µl (25 units) of MuLV Reverse Transcriptase in a final volume of 20 µl. Reverse transcription reactions were incubated in a PCR System 9700 Thermocycler (Applied Biosystems) using the following cycling parameters: 25^{o}C for 10 min, 42^{o}C for 60 min, 95^{o}C for 5 min (inactivation), and 4^{o}C hold. The subsequent quantitative PCR reaction was performed on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). PCR reactions contained 0.5 µl of cDNA template, 1X SYBR Green Buffer Master Mix and 2.5 pmol of forward and reverse primer in a final volume of 10 µl. Gene specific primer set sequences are as follows: *Cyp2b10* (Forward, 5'ACCCCACGTTCCTCTTCA3'; Reverse, 5'-CAGCAGGCGCAAGAACTGA-3'); *Cyp2c29* (Forward, 5'-GTATTTGGGCTCAAAGCCTACTGTCA-3'; Reverse, 5'-

CAGGGTCATGAGTGTAAATCGTCTCA-3'), *Cyp2c37* (Forward, 5'-GATGGCAATCAACCATTGC-3'; Reverse, 5'-GCCGATCACATGCTCAATT-3'), *Cyp3a11* (Forward, 5'-GTCAAACGCCTCTCCTTGCTG-3'; Reverse 5'-GGCTTGCCTTTCTTTGCCTTC-3'), and β-actin (Forward, 5'-CCTAGAAGCATTTGCGGTGCACGATG-3; Reverse, 5'-TCATGAAGTGTGACGTTGACATCCGT-3'). PCR cycling parameter were as follows: 50^{o}C for 2 min hold, 95^{o}C for 10 min hold, 94^{o}C for 30 sec (denaturation), 60^{o}C for 30 sec (annealing), and 72^{o}C for 30 sec (extension). Denaturation, annealing, and extension temperatures and times were repeated for 42 cycles. PCR products were analyzed using gel electrophoresis, dissociation curve analysis, and by dye terminator DNA sequencing (Applied Biosystems) to determine single product formation and gene specificity. Standard curves (log of template dilution vs Ct value) for each gene specific primer set were used to determine relative mRNA content for each target gene. Each gene specific PCR reaction was performed in triplicate within each pooled experimental group. The triplicate values obtained from each gene specific PCR reaction was used to determine a relative starting template amount mean and standard error for each of the experimental groups.

Immunoblot

Hepatic microsomes were prepared from frozen liver tissue from individual mice following a previous method described (Hahn et al., 1989). After comparison of murine Cyp2C sequences, a CYP2C37 specific peptide, CGQEDGNQPLQNR, was synthesized by Invitrogen (Carlsbad, CA). The specific peptide sequence was

coupled to keyhole limpet hemocyanin through the N-terminus cysteine. Custom polyclonal antibodies specific to CYP2C37 peptide were then produced by Covance Research Products Inc. (Princeton, NJ) as previously described (DeLozier et al., 2004). Hepatic microsomes and bacterial expressed partially purified recombinant CYP2C37 protein (Luo et al., 1998) were electrophoresed under reducing conditions using 10% Bis-Tris NuPAGE precast minigels (Invitrogen) in 1X NuPAGE MOPS SDS running buffer (Invitrogen). Proteins were then transferred to precut nitrocellulose membranes (Invitrogen) in 1X NuPAGE transfer buffer (Invitrogen) supplemented with 10% methanol. Membranes were immunoblotted with rabbit anti-CYP2C37 peptide-specific antibody (1:500) and donkey anti-rabbit IgG conjugated to horseradish peroxidase from Amersham Biosciences Inc. (Piscataway, NJ). Immunblots were stripped using Restore Western Blot Stripping Buffer (Pierce, Rockford, IL) to remove primary antibody and secondary antibody interactions. Stripping of the CYP2C37 immunoblot was performed by soaking the immunoblot in 20 ml of stripping buffer and incubating at 37^{o}C for 1 hour. The polyclonal antibodies specific to CYP2C37 protein were blocked using the specific CYP2C37 synthesized peptide used in antibody production. A 40 µl aliquot of CYP2C37 antiserum was incubated with a 40 µl aliquot of CYP2C37 (1 µg/µl) synthesized peptide for 1 hour at room temperature to block CYP2C37 antiserum. Protein bands were visualized using SuperSignal West Pico Chemiluminescent Substrate from Pierce and Biomax MR Film (Kodak, Rochester, NY).

Electrophoretic mobility Shift Assay (EMSA)

EMSA was performed on a 5% polyacrylamide gel using 0.5X TBE running buffer. Oligonucleotides were labeled with [α-^{32}P]dCTP and probe was purified by Microspin G-25 columns (Amersham Biosciences) to remove unincorporated dNTP's. Oligonucleotide sequences used in EMSA were as follows: *CYP2C9* -1839 CAR-RE (Forward, 5'-CTAGACCAAACTCTTCTGACCTCT-3'; Reverse, 5'-CTAGAGAGGTCAGAAGAGTTTGGT-3'); *Cyp2c37* -1890 CAR-RE (Forward, 5'-CTAGAGTTCTCTCCTGGATGAATTTGGGT-3'; Reverse, 5'-CTAGACCCAAATTCATCCAGGAGAGAACT-3'); *Cyp2c37* -2065 CAR-RE (Forward, 5'-CTAGGTTACTGTGCTGGGTGAACTGTGTT-3'; Reverse, 5'-CTAGAACACAGTTCACCCAGCACAGTAAC-3'); *Cyp2c37* -2791 CAR-RE (Forward, 5'-CTAGAAAAGCAAACTTTTCTGAACTCCATG; Reverse, 5'-CTAGCATGGAGTTCAGAAAAGTTTGCTTTT-3'); *Cyp2c37* -2820 CAR-RE (Forward, 5'-CTAGAGCCCGTATCACAAAGTTCAACAAG-3'; Reverse, 5'-CTAGCTTGTTGAACTTTGTGATACGGGCT-3'); *Cyp2c37* -3119 CAR-RE (Forward, 5'-CTAGATTAGTGAAATCAAAATGTGATGTATGAAATTCAAG-3'; Reverse, 5'-CTAGCTTGAATTTCATACATCACATTTTGATTTCACTAAT-3'); *Cyp2c37* -3205 CAR-RE (Forward, 5'-CTAGCAAATAGAACAACATAAACTGAGAC; Reverse, 5'-CTAGGTCTCAGTTTATGTTGTTCTATTTG3'). Labeled probe (~100,000 cpm per rxn) was applied to each binding reaction in 2 µl of 5X binding buffer, 0.5 µg/ul poly(dI-dC), and 1 µl of each *in vitro* transcribed/translated protein (mCAR and hRXR) in a final volume of 10 µl. 5X binding buffer was composed of 20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, and 50 mM Tris-HCl (pH

7.5). After addition of probe, the reactions were peformed at room temperature for 20 minutes before being loaded on a polyacrylamide gel and electrophoresed. Gels were then dried and exposed to film for 16 to 24 hours at -80^{o}C.

Expression Vectors and Cloning of the *Cyp2c37* 5'-Flanking Region

Author M.N provided the nuclear receptor expression plasmid pCR3-mCAR and pGEMT-hRXR. PCR was performed to isolate a 1.97 kb fragment of the *Cyp*2c37 5'-flanking sequence using the following primers: Forward, 5'-TGCTGTTAGCTGGTCTTGTTCTCTC-3'; Reverse, 5'-CCATGGAGATTCTTCTTACTGACACA-3'. The reverse primer introduced an Nco I restriction site at the 3' end of the PCR product. The PCR fragment was generated using mouse genomic DNA from Promega (Madison, WI). This fragment was then subcloned into pCR2.1 Topo TA vector (Invitrogen) following manufacturer's protocol. The endonucleases Bgl II and Nco I were used to remove a 1.8 kb fragment of the *Cyp2c37* 5'-flanking region from the pCR2.1 Topo TA subclone. This 1.8 kb fragment was then ligated into a Bgl II and Nco I digested pGL3Basic luciferase reporter vector (Promega) producing the *Cyp2c37* -1.8 kb luciferase reporter. The *Cyp2c37* 5'-flanking sequence from -1842 bp to -2887 bp was amplified from murine genomic DNA (Promega) by PCR using forward primer 5'-ACGCGTGGGAAATATAGGGCAATGTGCATC-3' and reverse primer 5'-GAAGATCTATAGCACAGGGTCAGCTC-3'. The forward primer introduced a Mlu I site in the 5' end of the PCR product for *Cyp2c37* 5'-flanking sequence. This ~1Kb fragment was then subcloned into pCR2.1 Topo TA vector (Invitrogen), then

digested by Bgl II to excise an ~876 bp fragment using interior Bgl II sites, and finally ligated into a Bgl II digested *Cyp2c37* -1.8 kb luciferase reporter to create the *Cyp2c37* -2.7 kb luciferase reporter. The subclone for -1842 bp to -2887 bp *Cyp2c37* 5'-flanking sequence was digested by Stu I and Mlu I to remove an ~273 bp fragment (-2887 bp to -2614 bp). Subsequent ligation of this fragment into a Stu I and Mlu I digested *Cyp2c37* -2.7 kb luciferase reporter created the *Cyp2c37* -2.9 kb luciferase reporter. Further amplification of the *Cyp2c37* 5'-flanking sequence was needed to create the *Cyp2c37* -3.6 kb luciferase reporter. Again, murine genomic DNA (Promega) was used as a template to amplify *Cyp2c37* 5'-flanking sequence from -2540 bp to -3598 bp using a forward primer 5'-CGCGTTTGTAGAGTTAGAAAACACAATTTGC-3' introducing a Mlu I site and reverse primer 5'-CCCAGTTTCTCATGCTCAAATGGAA-3'. The PCR product -2540 bp to -3598 bp of the *Cyp2c37* 5'-flanking sequence was subcloned into pCR2.1 Topo TA vector (Invitrogen) and then digested with Mlu I and Stu I to excise an ~1050 bp fragment of *Cyp2c37* 5'-flanking sequence. This fragment was then ligated into a Stu I and Mlu I digested Cyp2c37 -2.9 kb luciferase reporter, producing the *Cyp2c37* -3.6 kb luciferase reporter. Mutations of putative binding sites in *Cyp2c37* luciferase reporters were produced by site-directed mutagenesis using QuikChange (Stratagene, La Jolla, CA) on the *Cyp2c37* -2.9 kb luciferase reporter background. Mutant oligonucleotides used in the site-directed mutagenesis reactions are as follows: -2791Bmut forward, 5'-ATAAAAGCAAACTTTTCCCCCCTCCATGCAATAAAAACAGTG-3'; -2791Bmut reverse, 5'-CACTGTTTTTATTGCATGGAGCCCCGAAAAGTTTGCTTTTAT-3'; -

2791Amut forward, 5'-CAAGTTATAAAAGCCCCCCTTTCCCCCCTCCATGC-3'; -2791Amut reverse, 5'-GCATGGAGGGGGGAAAGGGGGGCTTTTATAACTTG-3'. (mutations underlined) Dye terminator DNA sequencing (Applied Biosystems) was used to verify all sequences.

Transcriptional Activation Assays

HepG2 cells were maintained in Eagles minimum essential media (Invitrogen) supplemented with 10% fetal bovine serum and 1X penicillin-streptomycin-glutamine solution (Invitrogen). Cells were plated into 24 well plates at a density of ~100,000 cells/well. Transfections included 100 ng of nuclear receptor (pCR3-mCAR), 100 ng of each luciferase promoter construct, and 1 ng of pRL-tk transfection control. Twenty-four hours after transfection, cells were treated with drugs or vehicle and incubated for an additional 24 hours. Cells were subsequently lysed with 100 µl of 1X passive lysis buffer (Promega) for 30 min at room temperature with gentle rocking, and dual luciferase assays (Promega) were then performed on cell lysates per manufacturer's procedures.

3.4 Results

Induction of Hepatic CYP2C37 mRNA and Protein in Wild-type Mice

Wild-type mice were initially treated with corn oil (10 ml/kg), phenobarbital (80 mg/kg), or phenytoin (80mg/kg) via oral gavage for four consecutive days. CYP2C37 mRNA was induced ~10 fold by phenobarbital and ~20 fold by phenytoin (Fig. 1). Consistent with our previous findings (Jackson et al., 2004), CYP2C29 mRNA was also induced ~3 fold by phenobarbital and ~4 fold by phenytoin.

Hepatic microsomes prepared from individual mice were subjected to immunoblot analysis to determine hepatic CYP2C37 protein content before and after phenytoin treatment using a CYP2C37 peptide antibody. Several bands in the 50 kD region were recognized by this antibody including the partially purified recombinant CYP2C37 standard in lane 7 (~ 50 kD). To determine which immunoreactive bands were CYP2C37 specific, CYP2C37 antiserum was blocked with the specific CYP2C37 peptide and used to reprobe the stripped immunoblot. Comparison of blots (Fig 2A and 2B) indicates that the ~45 kD immunoreactive bands (denoted by arrow) are CYP2C37 specific as indicated by the absence of these bands in the peptide blocked CYP2C37 antiserum blot B. The CYP2C37 standard was also absent in blot B indicating that the peptide inhibited the specific CYP2C37 immunoreactivity of the polyclonal antibody. Although, the electrophoretic mobility of the recombinant CYP2C37 protein and the microsomal protein identified as CYP2C37 differed, the molecular weight of bacterial expressed partially purified recombinant P450 standards almost invariably differs from their native P450 counterparts in our experience due to the N-terminal modification of the cDNA,

which is performed to maximize the expression of P450 enzymes in Escherichia coli expression systems (Barnes, 1996). These blots were repeated twice with identical results.

Element Identification and Binding Analysis

The identification of putative CAR and PXR response elements was performed using SeqLab GCG (Accelrys, San Diego, CA), a soft-ware based analysis tool, to search 10 kb of *Cyp2c37* 5'-flanking region for imperfect direct repeats of AGGTCA spaced by 3 to 5 nucleotides (DR-n). Five DR-3 motifs located at -3205 bp, -3102 bp, -2820 bp, -2065 bp, and -1890 bp; one DR-5 motif located at -3113 bp; and one DR-4 motif located at -2791 bp were identified as putative response elements within 3.6 kb from the start of *Cyp2c37* translation. Electrophoretic mobility shift assays (EMSA) were performed to determine if mCAR could bind any of the aforementioned putative response elements. EMSA results indicated that the putative DR-3 CAR-RE's, located at -3205 bp, -3102 bp, -2820 bp, -2065 bp, and -1890 bp from the start of *Cyp2c37* translation, did not bind mCAR (data not shown). EMSA results also showed that the one putative DR-5 motif, located at -3113 bp from the start of *Cyp2c37* translation, did not bind mCAR (data not shown). However, EMSA results demonstrated that the putative DR-4 response element, located at -2791 bp from start of *Cyp2c37* translation, bound mCAR strongly (Fig. 3A). This interaction was reduced with 25 fold molar excess of nonradiolabeled oligonucleotides. The *Cyp2c37* DR-4 CAR response element

(CAR-RE) has high sequence homology with a known human *CYP2C9* CAR binding site at -1839 bp. (Fig. 3B).

Cyp2c37 5'-flanking Sequence Analysis

Various deletion and mutant Cyp2c37 5'-flanking region luciferase reporter plasmids were developed to identify CAR response regions within the Cyp2c37 5'-flanking sequence. These reporters were then cotransfected with mCAR into HepG2 cells and treated with mCAR activity modulators to identify mCAR specific transactivation. Cyp2c37 -1.8 kb and -2.7 kb luciferase reporters showed no mCAR constitutive transactivation (Fig. 4). However, the Cyp2c37 -2.9 kb and the Cyp2c37 -3.6 kb luciferase reporters were transactivated ~3 fold by mCAR consistent with our EMSA data. The constitutive transactivation of the -2.9 kb and the -3.6 kb Cyp2c37 luciferase reporters was repressed by the addition of 10 μM androstenol (Forman et al., 1998). Repression of mCAR constitutive transactivation could be reversed by the addition of 250 nM TCPOBOP, a mCAR specific activator (Tzameli et al., 2000)(Fig. 4). Mutagenesis was performed to determine if the Cyp2c37 -2791 CAR-RE site is necessary for mCAR constititutive transactivation of the Cyp2c37 -2.9 kb luciferase reporter. Mutagenesis of the "b" half-site of the CAR-RE (Fig. 3B) did not completely eliminate mCAR transactivation of the Cyp2c37 -2.9 kb luciferase reporter (Fig. 5). However, mCAR transactivation of the Cyp2c37 -2.9 kb luciferase reporter was completely abolished by simultaneous mutations of the "a" and "b" half-sites of the Cyp2c37 -2791 CAR-RE (Fig. 6). Furthermore, the -2791 CAR-RE double mutant luciferase reporter and Cyp2c37 -2.7 kb luciferase reporter

were unresponsive to TCPOBOP and androstenol treatment in the presence of mCAR (Fig. 6).

Evaluation of Phenytoin Induction in CAR-null and PXR-null mice

We used CAR-null and PXR-null mice to examine whether induction of hepatic CYP2B10, CYP2C29, CYP2C37, and CYP3A11 mRNA by phenytoin is mediated by CAR or PXR. In wild-type congenic mice strains for both the CAR-null and PXR-null mice, all P450 genes examined were induced by phenytoin and phenobarbital (Fig. 7 & 8). In CAR-null mice, phenytoin and phenobarbital induction of CYP2B10 mRNA was completely abolished, as expected (Fig. 7). The induction of CYP2C29 and CYP2C37 mRNA by phenobarbital in CAR-null mice was drastically reduced ~98% ($p<0.001$) and ~87% ($p<0.001$), respectively, compared with the wild-type response. Similarly, phenytoin induction of CYP2C29 and CYP2C37 mRNA was severely decreased in CAR-null mice ~99% ($p<0.001$) and ~92% ($p<0.001$), respectively (Fig. 7). However, phenytoin induction of CYP3A11 mRNA was only moderately decreased (~66%, $p<0.01$) in CAR-null mice (Fig. 7). The mouse PXR agonist PCN induced CYP3A11 mRNA ~6 fold ($p<0.001$) in wild-type mice, but did not induce CYP2C29, CYP2C37, or CYP2B10 mRNA (Fig. 8). Induction of CYP3A11 mRNA by PCN was abolished in PXR-null mice consistent with its known PXR mediated induction (Xie et al., 2000; Staudinger et al., 2001a; Staudinger et al., 2001b; Goodwin et al., 2002) (Fig. 8). In PXR-null mice, induction of CYP2B10, CYP2C29, and CYP2C37 mRNA by phenytoin was reduced moderately but not abolished (Fig. 8). Phenytoin induction of CYP3A11 mRNA in

PXR-null mice was reduced ~45% ($p<0.001$) compared with the response observed in wild-type mice (Fig. 8). Taken together, these data suggest that CYP2B10, CYP2C29 and CYP2C37 induction by phenytoin is mediated primarily by CAR. In contrast, PCN, a known PXR agonist, does not induce these enzymes. The induction of CYP3A11 by phenytoin appears to be mediated by both receptors as suggested by CAR-null and PXR-null mice data.

The constitutive expression of CYP2C29 mRNA was severely reduced ~68 fold ($p=0.02$) in CAR-null mice (Fig. 7). This observation is consistent with our previously reported findings (Jackson et al., 2004). However, constitutive expression of CYP2C29 mRNA was not appreciably altered in PXR-null mice (Fig. 8).

3.5 Discussion

We have identified a second phenytoin inducible murine CYP2C gene, *Cyp2c37*. Using CAR-null and PXR-null mice, we demonstrated that CYP2C37 mRNA induction by phenytoin is primarily CAR mediated. A functional DR-4 CAR-RE was identified at -2791 bp from the translation start site of the *Cyp2c37* gene. Furthermore, we examined phenytoin induction of CYP2B10, CYP2C29 and CYP3A11 mRNA in CAR-null and PXR-null mice to determine if CAR solely mediated the induction of these murine genes by phenytoin.

Previously, we reported that the nuclear receptor CAR mediated phenytoin induction of the *Cyp2c29* gene (Jackson et al., 2004), thus we searched the 5'-flanking sequence (~10 kb) of *Cyp2c37* for putative nuclear receptor response elements. We identified several of these elements located within -3.6 kb of the *Cyp2c37* translation start site (Fig. 9A). However, only the putative DR-4 CAR-RE located at -2791 bp was demonstrated to bind mCAR strongly. Interestingly, Cyp2c37 -2791 CAR-RE shares high-sequence homology with the -1839 CAR-RE discovered in human CYP2C9 (Ferguson et al., 2002; Gerbal-Chaloin et al., 2002).

Transient transfections in HepG2 cells with mCAR and the -1.8, -2.7, -2.9, or -3.6 kb *Cyp2c37* luciferase reporters demonstrated that only the -2.9 and -3.6 kb luciferase reporters were transactivated by mCAR, ~3 fold. These data suggested that no CAR responsive regions were within -2.7 kb of the *Cyp2c37* translation start site, consistent with the identification of a putative CAR-RE at ~2.8 kb upstream of *Cyp2c37* translation start site. The -2.9 and -3.6 kb *Cyp2c37* luciferase reporters were equally activated by mCAR suggesting that the CAR-RE located at -2791 bp

from the *Cyp2c37* translation start site is the primary and essential CAR-RE. The transactivation of the -2.9 and -3.6 kb *Cyp2c37* luciferase reporters by mCAR was effectively repressed by androstenol, a mCAR specific inverse agonist (Forman et al., 1998). The repressed transactivation of the -2.9 and -3.6 kb *Cyp2c37* luciferase reporters could be reversed by the addition of TCPOBOP, a high affinity mCAR specific agonist (Sueyoshi et al., 1999; Tzameli et al., 2000). These results are typical for CAR regulated genes (Honkakoski et al., 1998b; Honkakoski and Negishi, 2000; Sueyoshi and Negishi, 2001; Chen et al., 2003; Jackson et al., 2004).

Site-directed mutagenesis of the "b" half-site of the -2791 CAR-RE reduced mCAR constitutive transactivation of the -2.9 kb *Cyp2c37* luciferase reporter to ~2 fold, but mutagenesis of both "a" and "b" half-sites of the -2791 CAR-RE completely abolished mCAR constitutive transactivation of the -2.9 kb *Cyp2c37* luciferase reporter. These data suggest that the -2791 CAR-RE is necessary for mCAR transactivation of the -2.9 kb *Cyp2c37* luciferase reporter. The single half-site "b" mutation was observed to be insufficient to abolish mCAR transactivation and may be explained by CAR binding as a monomer and acting as a fully functional transcription factor as reported by Frank et al. (2003). Our results are consistent with previous studies examining transcriptional regulation of the CYP2C subfamily by CAR such as human *CYP2C8*, *CYP2C9*, *CYP2C19*, and murine *Cyp2c29 (Ferguson et al., 2002; Gerbal-Chaloin et al., 2002; Chen et al., 2003; Chen et al., 2004; Jackson et al., 2004; Ferguson et al., 2005)*. In contrast, only one active CAR-RE was identified as the principle element conferring CAR responsiveness in the human genes CYP2C8 and CYP2C19 (Chen et al., 2003; Ferguson et al., 2005).

Similarly, *Cyp2c37* -2791 CAR-RE is the principle CAR responsive element within -2.9 kb of the *Cyp2c37* translation start site as indicated by mutagenesis studies.

Although there are species differences, the nuclear receptors CAR and PXR are known to overlap in function as xenobiotic sensors of a wide range of compounds (Honkakoski and Negishi, 2000; Makinen et al., 2002; Wei et al., 2002; Pascussi et al., 2003; Pascussi et al., 2004; Zhang et al., 2004). Many of the same xenobiotic detoxifying target genes are induced by these nuclear receptors via the same response elements (Pascussi et al., 2003; Pascussi et al., 2004). Redundancy can largely explain the cross-interaction of these distinct signaling pathways via two distinct mechanisms: (1) CAR and PXR bind the same response elements, and (2) CAR and PXR share some ligands/activators (Moore et al., 2000; Pascussi et al., 2003; Zhang et al., 2004). Thus in the absence of one receptor, many of the same detoxifying pathways are up-regulated.

The compounds dieldrin and clotrimazole have been shown to induce the murine genes *Cyp2b10* and *Cyp3a11* in wild-type mice (Wei et al., 2002; Zhang et al., 2004). In CAR-null mice, induction of CYP2B10 mRNA by either compound is abolished; clearly demonstrating that CYP2B10 induction is mediated by mCAR (Wei et al., 2002; Zhang et al., 2004). However, induction of CYP3A11 mRNA by dieldrin or clotrimazole is apparently mediated by both receptors since it is only abolished in CAR/PXR double null mice (Zhang et al., 2004). Phenytoin has been shown to induce human *CYP3A4*, but the low PXR-mediated increase in CYP3A4 promoter luciferase activity was inconsistent with high inducibility of the mRNA (Raucy et al., 2002). Recently two studies showed that CAR mediates phenytoin

induction of human *CYP2B6* gene and murine genes, *Cyp2c29* and *Cyp2b10* (Jackson et al., 2004; Wang et al., 2004). CYP2B10 and CYP2C29 mRNA is induced by phenytoin in wild-type mice and induction was essentially abolished in CAR-null mice. Furthermore, we verified that phenytoin induced the nuclear accumulation of mCAR in a manner similar to phenobarbital.

However, it is not clear whether phenytoin is a pure CAR agonist or a mixed CAR/PXR agonist for all genes. In this report, we tested these possibilities by examining hepatic CYP2B10, CYP2C29, CYP2C37, and CYP3A11 mRNA in CAR-null and PXR-null mice. CYP3A11 was used as an example of a gene that is induced by CAR and PXR agonists (Wei et al., 2002; Zhang et al., 2004). Induction of CYP2B10 by phenytoin was completely abolished in CAR-null mice, but remained in PXR-null mice. Similarly, CYP2C29 and CYP2C37 induction by phenytoin in CAR-null mice was severely reduced ~99% and ~92%, respectively, compared with the responses in wild-type mice. Although phenytoin induction of CYP2C29 and CYP2C37 were reduced moderately in PXR-null mice, a significant amount of induction remained. These moderate changes could suggest that PXR may mediate phenytoin induction of CYP2C29 and CYP2C37 in the absence of CAR or that these changes may be due to secondary effects in the PXR-null strain. Our results clearly indicate that murine CYP2C induction by phenytoin is primarily mediated by CAR. Interestingly, neither CYP2C37 nor CYP2C29 was induced by PCN, a mouse PXR agonist. PCN induction of CYP3A11 mRNA was completely removed in PXR-null mice consistent with PXR mediated induction (Xie et al., 2000; Staudinger et al., 2001a; Staudinger et al., 2001b; Goodwin et al., 2002). In contrast, the induction of

murine CYP3A11 mRNA by phenytoin was muted, but not abolished, in both single-null mice strains. These results suggest that the induction of CYP3A11 by phenytoin may be mediated by both receptors, and that phenytoin is a mixed CAR/PXR agonist similar to dieldrin and clotrimazole (Fig. 9B). Although double null mice in conjunction with single null mice would have clearly been desirable to examine whether induction of CYP3A11 by phenytoin is mediated both receptors, these mice are not available to our laboratory at this time.

In conclusion, we have demonstrated that CYP2C37 mRNA is induced by phenobarbital and phenytoin and that CYP2C37 protein is induced by phenytoin. We have also shown that the induction of hepatic CYP2C37 mRNA by phenobarbital and phenytoin is CAR-dependent. Furthermore, we identified a functional DR-4 CAR-RE located at -2791 bp from the *Cyp2c37* translation start site, which mediates CAR-dependent drug induction of the *Cyp2c37* gene. Additionally, our studies suggest that phenytoin activates both CAR and PXR similar to dieldrin and clotrimazole; thus transactivating P450 genes in a promoter dependent manner.

3.6 References Cited

Barnes HJ (1996) Maximizing expression of eukaryotic cytochrome P450s in Escherichia coli. *Methods Enzymol* 272:3-14.

Chen Y, Ferguson SS, Negishi M and Goldstein JA (2003) Identification of constitutive androstane receptor and glucocorticoid receptor binding sites in the CYP2C19 promoter. *Mol Pharmacol* 64:316-324.

Chen Y, Ferguson SS, Negishi M and Goldstein JA (2004) Induction of human CYP2C9 by rifampicin, hyperforin, and phenobarbital is mediated by the pregnane X receptor. *J Pharmacol Exp Ther* 308:495-501.

DeLozier TC, Tsao CC, Coulter SJ, Foley J, Bradbury JA, Zeldin DC and Goldstein JA (2004) CYP2C44, a new murine CYP2C that metabolizes arachidonic acid to unique stereospecific products. *J Pharmacol Exp Ther* 310:845-854.

Ferguson SS, Chen Y, LeCluyse EL, Negishi M and Goldstein JA (2005) Human CYP2C8 is transcriptionally regulated by the nuclear receptors constitutive androstane receptor, pregnane X receptor, glucocorticoid receptor, and hepatic nuclear factor 4alpha. *Mol Pharmacol* 68:747-757.

Ferguson SS, LeCluyse EL, Negishi M and Goldstein JA (2002) Regulation of human CYP2C9 by the constitutive androstane receptor: discovery of a new distal binding site. *Mol Pharmacol* 62:737-746.

Forman BM, Tzameli I, Choi HS, Chen J, Simha D, Seol W, Evans RM and Moore DD (1998) Androstane metabolites bind to and deactivate the nuclear receptor CAR-beta. *Nature* 395:612-615.

Frank C, Gonzalez MM, Oinonen C, Dunlop TW and Carlberg C (2003) Characterization of DNA complexes formed by the nuclear receptor constitutive androstane receptor. *J Biol Chem* 278:43299-43310.

Gerbal-Chaloin S, Daujat M, Pascussi JM, Pichard-Garcia L, Vilarem MJ and Maurel P (2002) Transcriptional regulation of CYP2C9 gene. Role of glucocorticoid receptor and constitutive androstane receptor. *J Biol Chem* 277:209-217.

Gerbal-Chaloin S, Pascussi JM, Pichard-Garcia L, Daujat M, Waechter F, Fabre JM, Carrere N and Maurel P (2001) Induction of CYP2C genes in human hepatocytes in primary culture. *Drug Metab Dispos* 29:242-251.

Goldstein JA and Blaisdell J (1996) Genetic tests which identify the principal defects in CYP2C19 responsible for the polymorphism in mephenytoin metabolism. *Methods Enzymol* 272:210-218.

Goodwin B, Hodgson E and Liddle C (1999) The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. *Mol Pharmacol* 56:1329-1339.

Goodwin B, Hodgson, E., D"Costa, D.J., Robertson, G.R., Liddle, C. (2002) Transcriptional Regulation of the Human *CYP3A4* Gene by the Constitutive Androstane Receptor. *Molecular Pharmacology* 62:359-365.

Goodwin B, Redinbo MR and Kliewer SA (2002) Regulation of cyp3a gene transcription by the pregnane x receptor. *Annu Rev Pharmacol Toxicol* 42:1-23.

Hahn ME, Goldstein JA, Linko P and Gasiewicz TA (1989) Interaction of hexachlorobenzene with the receptor for 2,3,7,8-tetrachlorodibenzo-p-dioxin in vitro and in vivo. Evidence that hexachlorobenzene is a weak Ah receptor agonist. *Arch Biochem Biophys* 270:344-355.

Honkakoski P, Moore R, Washburn KA and Negishi M (1998a) Activation by diverse xenochemicals of the 51-base pair phenobarbital- responsive enhancer module in the CYP2B10 gene. *Mol Pharmacol* 53:597-601.

Honkakoski P and Negishi M (2000) Regulation of cytochrome P450 (CYP) genes by nuclear receptors. *Biochem J* 347:321-337.

Honkakoski P, Zelko I, Sueyoshi T and Negishi M (1998b) The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the CYP2B gene. *Mol Cell Biol* 18:5652-5658.

Jackson JP, Ferguson SS, Moore R, Negishi M and Goldstein JA (2004) The constitutive active/androstane receptor regulates phenytoin induction of Cyp2c29. *Mol Pharmacol* 65:1397-1404.

Johnson EF, Hsu MH, Savas U and Griffin KJ (2002) Regulation of P450 4A expression by peroxisome proliferator activated receptors. *Toxicology* 181-182:203-206.

Kawamoto T, Sueyoshi T, Zelko I, Moore R, Washburn K and Negishi M (1999) Phenobarbital-responsive nuclear translocation of the receptor CAR in induction of the CYP2B gene. *Mol Cell Biol* 19:6318-6322.

LeCluyse EL (2001) Pregnane X receptor: molecular basis for species differences in CYP3A induction by xenobiotics. *Chem Biol Interact* 134:283-289.

Luo G, Zeldin DC, Blaisdell JA, Hodgson E and Goldstein JA (1998) Cloning and expression of murine CYP2Cs and their ability to metabolize arachidonic acid. *Arch Biochem Biophys* 357:45-57.

Makinen J, Frank C, Jyrkkarinne J, Gynther J, Carlberg C and Honkakoski P (2002) Modulation of mouse and human phenobarbital-responsive enhancer module by nuclear receptors. *Mol Pharmacol* 62:366-378.

Meyer RP, Hagemeyer CE, Knoth R, Kurz G and Volk B (2001) Oxidative hydrolysis of scoparone by cytochrome p450 CYP2C29 reveals a novel metabolite. *Biochem Biophys Res Commun* 285:32-39.

Moore LB, Parks DJ, Jones SA, Bledsoe RK, Consler TG, Stimmel JB, Goodwin B, Liddle C, Blanchard SG, Willson TM, Collins JL and Kliewer SA (2000) Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. *J Biol Chem* 275:15122-15127.

Pascussi JM, Gerbal-Chaloin S, Drocourt L, Assenat E, Larrey D, Pichard-Garcia L, Vilarem MJ and Maurel P (2004) Cross-talk between xenobiotic detoxication and other signalling pathways: clinical and toxicological consequences. *Xenobiotica* 34:633-664.

Pascussi JM, Gerbal-Chaloin S, Drocourt L, Maurel P and Vilarem MJ (2003) The expression of CYP2B6, CYP2C9 and CYP3A4 genes: a tangle of networks of nuclear and steroid receptors. *Biochim Biophys Acta* 1619:243-253.

Raucy JL, Mueller L, Duan K, Allen SW, Strom S and Lasker JM (2002) Expression and induction of CYP2C P450 enzymes in primary cultures of human hepatocytes. *J Pharmacol Exp Ther* 302:475-482.

Squires EJ, Sueyoshi T and Negishi M (2004) Cytoplasmic localization of pregnane X receptor and ligand-dependent nuclear translocation in mouse liver. *J Biol Chem* 279:49307-49314.

Staudinger J, Liu Y, Madan A, Habeebu S and Klaassen CD (2001a) Coordinate regulation of xenobiotic and bile acid homeostasis by pregnane X receptor. *Drug Metab Dispos* 29:1467-1472.

Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, Reinhard J, Willson TM, Koller BH and Kliewer SA (2001b) The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc Natl Acad Sci U S A* 98:3369-3374.

Sueyoshi T, Kawamoto T, Zelko I, Honkakoski P and Negishi M (1999) The repressed nuclear receptor CAR responds to phenobarbital in activating the human CYP2B6 gene. *J Biol Chem* 274:6043-6046.

Sueyoshi T and Negishi M (2001) Phenobarbital response elements of cytochrome P450 genes and nuclear receptors. *Annu Rev Pharmacol Toxicol* 41:123-143.

Tzameli I, Pissios P, Schuetz EG and Moore DD (2000) The xenobiotic compound 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene is an agonist ligand for the nuclear receptor CAR. *Mol Cell Biol* 20:2951-2958.

Wang H, Faucette S, Moore R, Sueyoshi T, Negishi M and LeCluyse E (2004) Human constitutive androstane receptor mediates induction of CYP2B6 gene expression by phenytoin. *J Biol Chem* 279:29295-29301.

Wang H, Faucette S, Sueyoshi T, Moore R, Ferguson S, Negishi M and LeCluyse EL (2003) A novel distal enhancer module regulated by pregnane X receptor/constitutive androstane receptor is essential for the maximal induction of CYP2B6 gene expression. *J Biol Chem* 278:14146-14152.

Wei P, Zhang J, Dowhan DH, Han Y and Moore DD (2002) Specific and overlapping functions of the nuclear hormone receptors CAR and PXR in xenobiotic response. *Pharmacogenomics J* 2:117-126.

Xie W, Barwick JL, Downes M, Blumberg B, Simon CM, Nelson MC, Neuschwander-Tetri BA, Brunt EM, Guzelian PS and Evans RM (2000) Humanized xenobiotic response in mice expressing nuclear receptor SXR. *Nature* 406:435-439.

Zhang J, Huang W, Qatanani M, Evans RM and Moore DD (2004) The constitutive androstane receptor and pregnane X receptor function coordinately to prevent bile acid-induced hepatotoxicity. *J Biol Chem* 279:49517-49522.

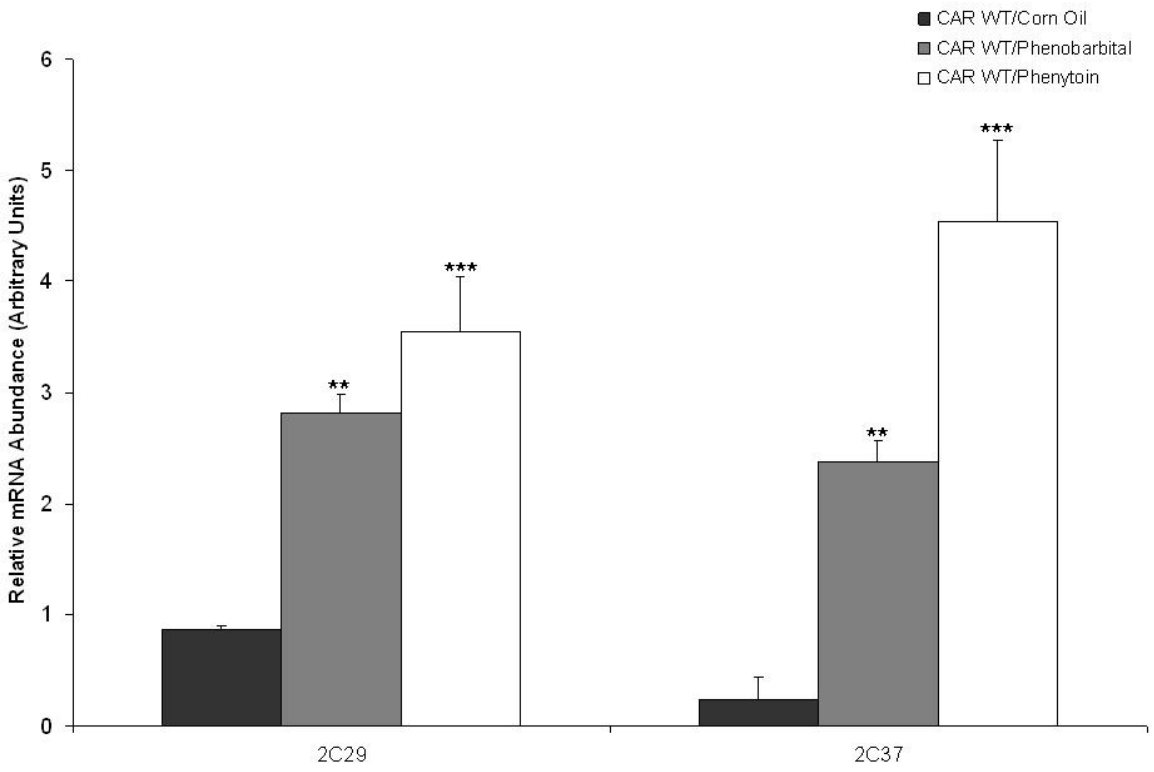

Figure 3.1- Drug response of hepatic CYP2C29 and CYP2C37 mRNA in C3H mice. Mice were treated via oral gavage with corn oil, phenobarbital (80 mg/kg), or phenytoin (80 mg/kg) for four consecutive days and sacrificed 24 hrs after last dose to remove liver for isolation of hepatic total RNA. Quantitative RT-PCR was performed to determine hepatic CYP2C29 and CYP2C37 mRNA content in response to phenobarbital and phenytoin. Target genes were normalized to a reference gene, β-actin. Values expressed above represent the relative abundance of gene specific mRNA +/- SE. P-values were determined using the Dunnett's Method comparing treated to corn oil control. ** $p \leq 0.01$ *** $p \leq 0.001$.

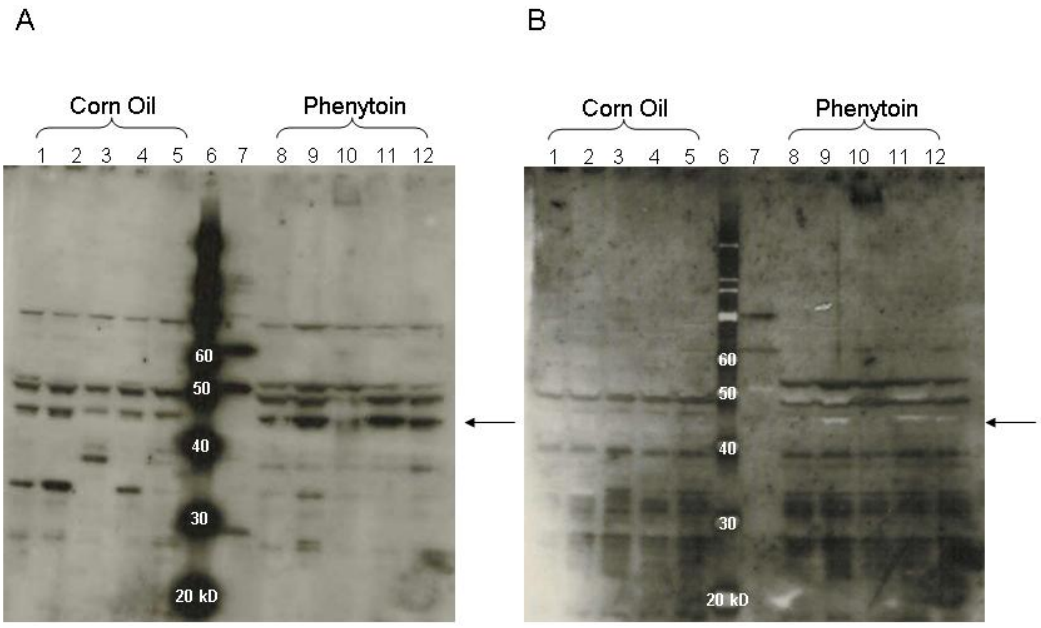

Figure 3.2- Induction of hepatic CYP2C37 protein in C3H wild-type mice. Mice were treated as previously described. Microsomes from individual livers (15 µg protein/lane) were analyzed on a 10% Bis/Tris NuPAGE gel (Invitrogen) and then transferred to a nitrocellulose membrane. CYP2C37 partially purified recombinant protein was used as a standard at 0.1 pmol, lane 7. A: Immunoblot using a CYP2C37 peptide polyclonal antibody B: Same blot stripped and reprobed with CYP2C37 peptide polyclonal antibody blocked with CYP2C37 specific peptide to identify specific CYP2C37 immunoreactive bands. Comparison of blots suggests that the ~45 kD bands (denoted by arrow) are immunoreactive to CYP2C37 as indicated by the absence of these bands in the peptide blocked CYP2C37 antiserum blot B.

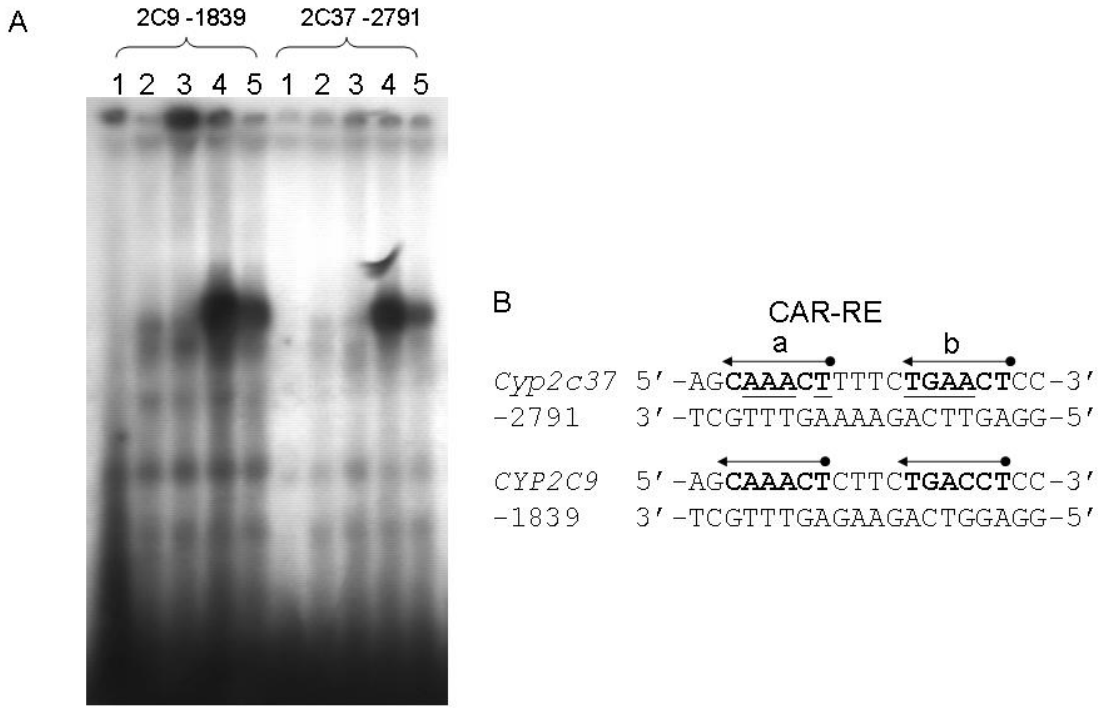

Figure 3.3- Analysis of mCAR binding to -2791 putative CAR-RE using EMSA. A: Radio-labeled -2791 putative CAR-RE (100,000 cpm ^{32}P) oligonucleotides were incubated with *in vitro* transcribed and translated mCAR, hRXR or mCAR/hRXR proteins. In parallel experiments, incubation was performed in the presence of 25 fold molar excess of nonradiolabeled probe. EMSA data demonstrated that the *Cyp2c37* putative -2791 CAR-RE binds mCAR/RXR as indicated by the presence of a band/shift in lane 3. This interaction could be specifically competed out using nonradiolabeled *Cyp2c37* -2791 CAR-RE oligonucleotides as demonstrated in lane 4. The positive control, *CYP2C9* -1839 CAR-RE, showed similar results. Lanes: (1) radiolabeled probe only (2) with mCAR only (3) with hRXR only (4) with mCAR/hRXR (5) with mCAR/hRXR & nonradiolabeled competitor. B: Illustration comparing *CYP2C9* -1839 CAR-RE sequence to that of *Cyp2c37* -2791 putative CAR-RE sequence. Underlined nucleotides in *Cyp2c37* -2791 putative CAR-RE sequence were mutated to deoxycytidines using site-directed mutagenesis (Stratagene) for use in luciferase reporter activity analyses.

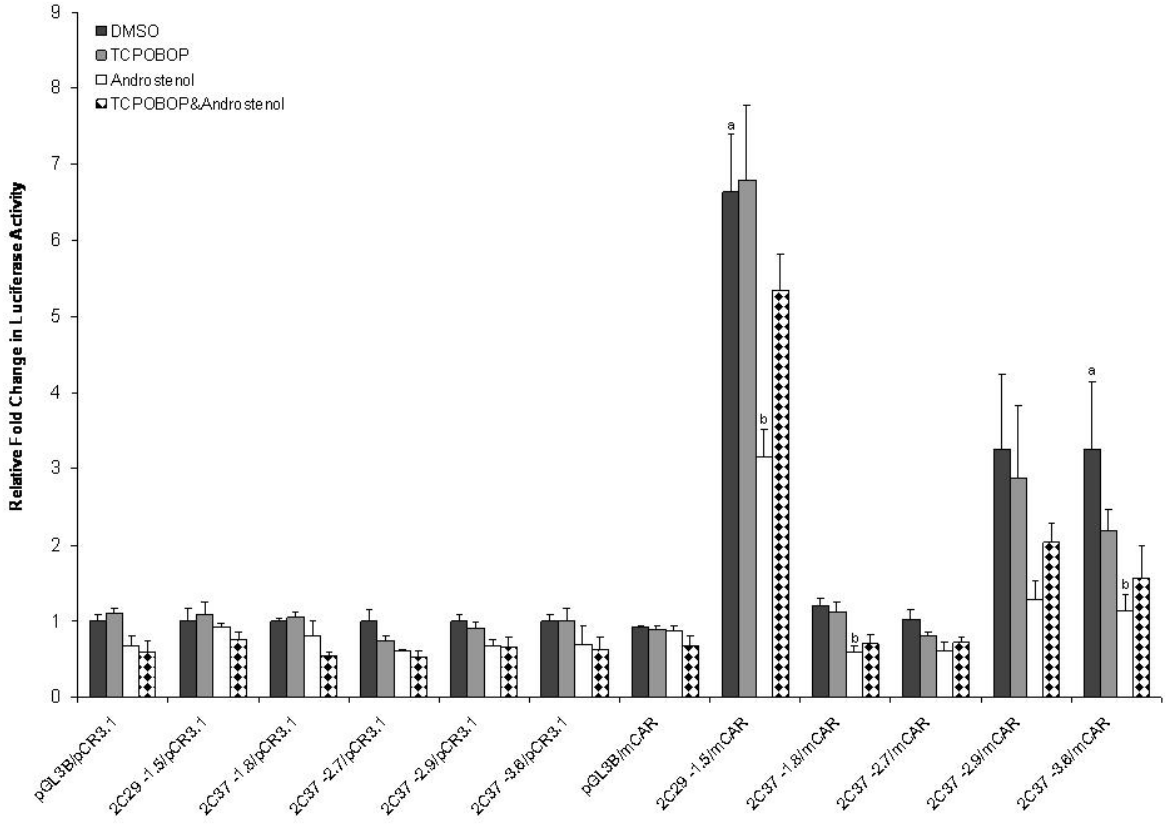

Figure 3.4- Transcriptional activation analysis of the *Cyp2c37* 5'-flanking region by mCAR. HepG2 cells were cotransfected with pRL-Tk (internal transfection control), expression vectors mCAR (pCR3) or empty (pCR3.1), and pGL3 Basic luciferase vectors containing varying lengths of the *Cyp2c37* 5'-flanking region to evaluate mCAR effects on gene reporter activity. These data represent the results of three independent transfections. *Cyp2c29* -1.5kb luciferase reporter (positive control) showed mCAR specific transactivation as indicated by mCAR alone, repression by 10 μM androstenol, and derepression by 250 nM TCPOBOP. P-values were determined using the Tukey-Kramer HSD Test. [a] *$p \leq 0.05$, mCAR significantly increased luciferase activity compared to no receptor DMSO control group.* [b] *$p \leq 0.05$, androstenol significantly decreased luciferase activity compared to mCAR DMSO treatment group.* [c] *$p \leq 0.05$, 250 nM TCPOBOP significantly derepressed mCAR repression by androstenol.*

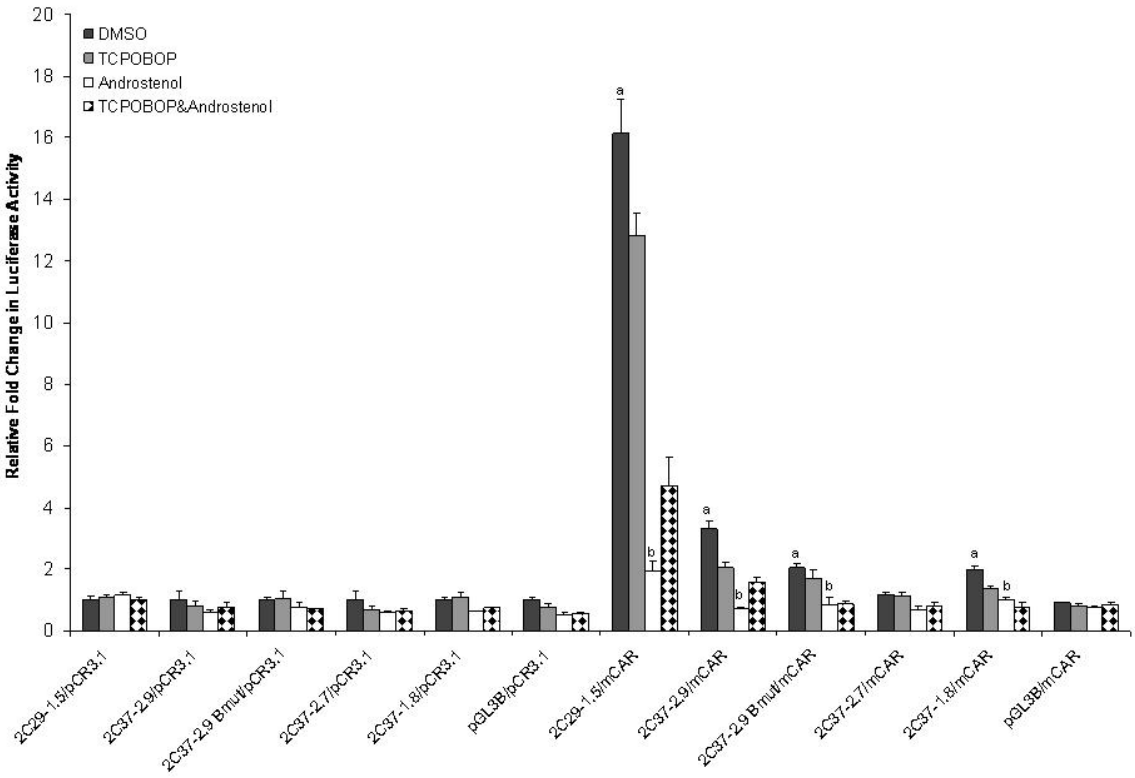

Figure 3.5- Functional analysis of the *Cyp2c37* -2791 CAR-RE. HepG2 cells were cotransfected with pRL-Tk (internal transfection control), expression vectors mCAR (pCR3) or empty (pCR3.1), and *Cyp2c37* wild-type or mutant luciferase reporters to evaluate mCAR effects on gene reporter activity. These data represent the results of three independent transfections. Mutation of -2791 imperfect DR-4 CAR-RE within the "b" half-site did not abolish mCAR transactivation and transactivation was effectively repressed by androstenol (10 μM). However, this mutation did reduce mCAR constitutive transactivation by one-third. P-values were determined using the Tukey-Kramer HSD Test. [a] *$p \leq 0.05$, mCAR significantly increased luciferase activity compared to no receptor DMSO control group.* [b] *$p \leq 0.05$, androstenol significantly decreased luciferase activity compared to mCAR DMSO treatment group.* [c] *$p \leq 0.05$, 250 nM TCPOBOP significantly derepressed mCAR repression by androstenol.*

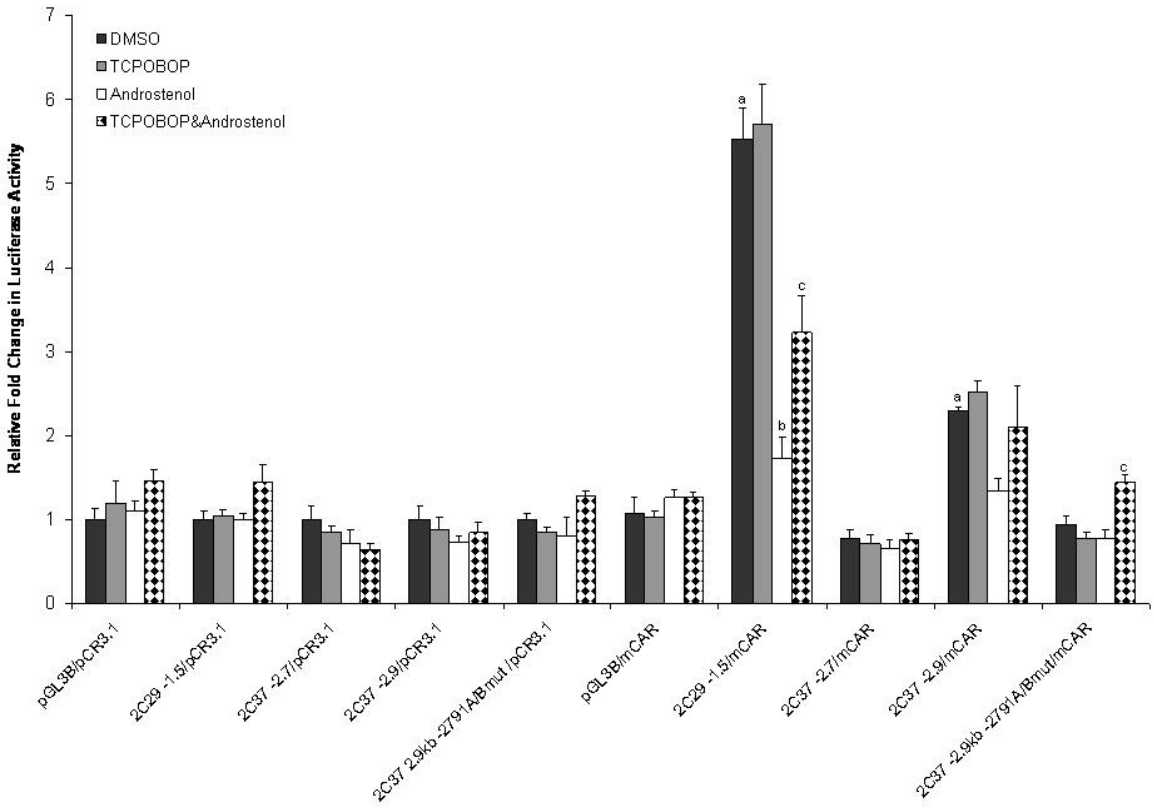

Figure 3.6- Functional analysis of the *Cyp2c37* -2791 A/B CAR-RE mutant. HepG2 cells were cotransfected with pRL-Tk (internal transfection control), expression vectors mCAR (pCR3) or empty (pCR3.1), and *Cyp2c37* wild-type or -2.9 kb Cyp2c37 A/B mutant luciferase reporter to evaluate mCAR effects on gene reporter activity. These data represent the results of three independent transfections. Mutation of -2791 imperfect DR-4 CAR-RE within the "a" and "b" half-sites abolished mCAR transactivation. This reporter was also not responsive to androstenol (10 μM) repression. P-values were determined using the Tukey-Kramer HSD Test. [a] *$p \leq 0.05$, mCAR significantly increased luciferase activity compared to no receptor DMSO control group.* [b] *$p \leq 0.05$, androstenol significantly decreased luciferase activity compared to mCAR DMSO treatment group.* [c] *$p < 0.05$, 250 nM TCPOBOP significantly derepressed mCAR repression by androstenol*.

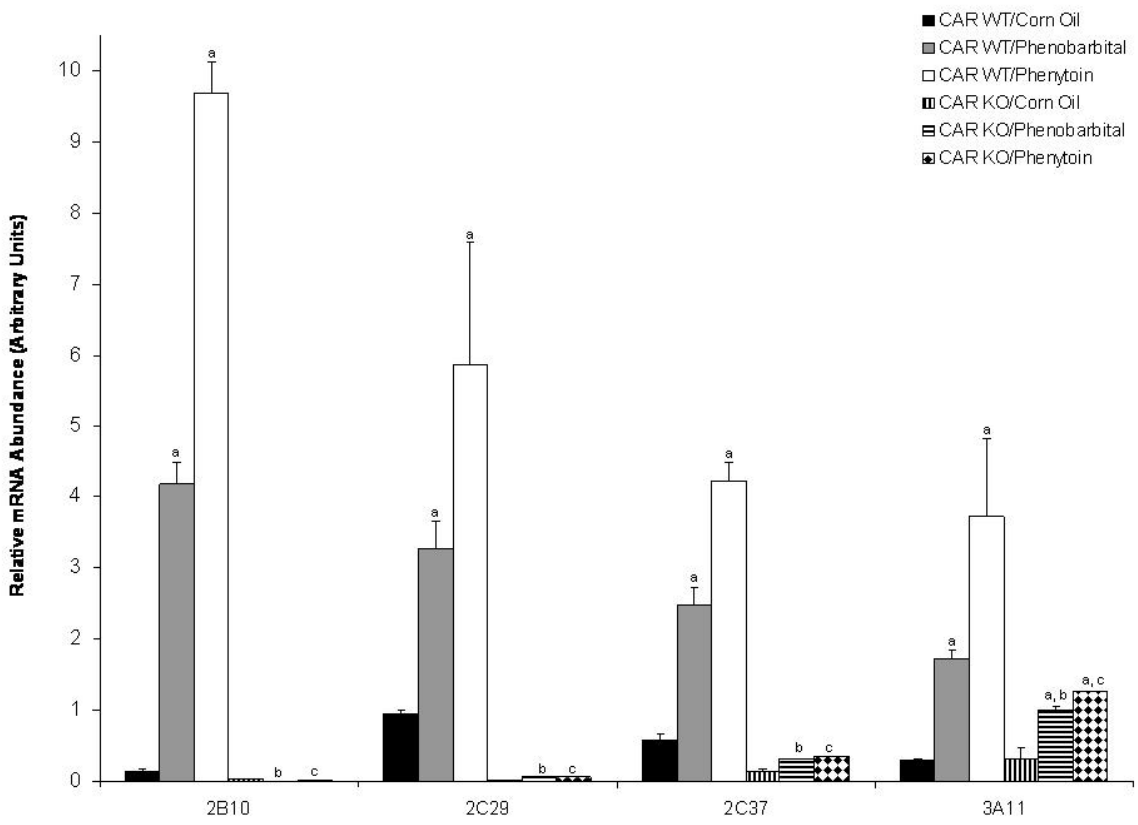

Figure 3.7- Induction of hepatic CYP2B10, CYP2C29, CYP2C37, and CYP3A11 mRNA in C3H wild-type and CAR-null mice. Mice were treated as previously described. Quantitative RT-PCR was performed to evaluate target gene mRNA content in response to phenobarbital and phenytoin. Target genes were normalized to a reference gene, β-actin. CYP2C29 and CYP2C37 mRNA was induced ~3 fold and ~4 fold by PB, respectively. Similarly, phenytoin induced CYP2C29 ~6 fold and CYP2C37 ~7 fold. In CAR-null mice, PB and phenytoin induction of CYP2C29 was severely reduced by ~98% and ~99%, respectively. PB and phenytoin Induction of CYP2C37 in CAR-null mice were also drastically reduced ~87% and ~92%, respectively. CYP3A11 mRNA was induced ~6 fold by PB and ~12 fold by phenytoin. In CAR-null mice, PB and phenytoin induction of CYP3A11 mRNA was reduced ~42% and ~66%. Values expressed above represent the relative abundance of gene specific mRNA +/- SE. P-values were determined using a LSMeans pairwise contrast comparison with a Bonferroni correction. [a] *p< 0.01, significantly higher than corn oil controls.* [b] *p < 0.01, significantly lower than wild-type treated with phenobarbital.* [c] *p < 0.01, significantly lower than wild-type treated with phenytoin*.

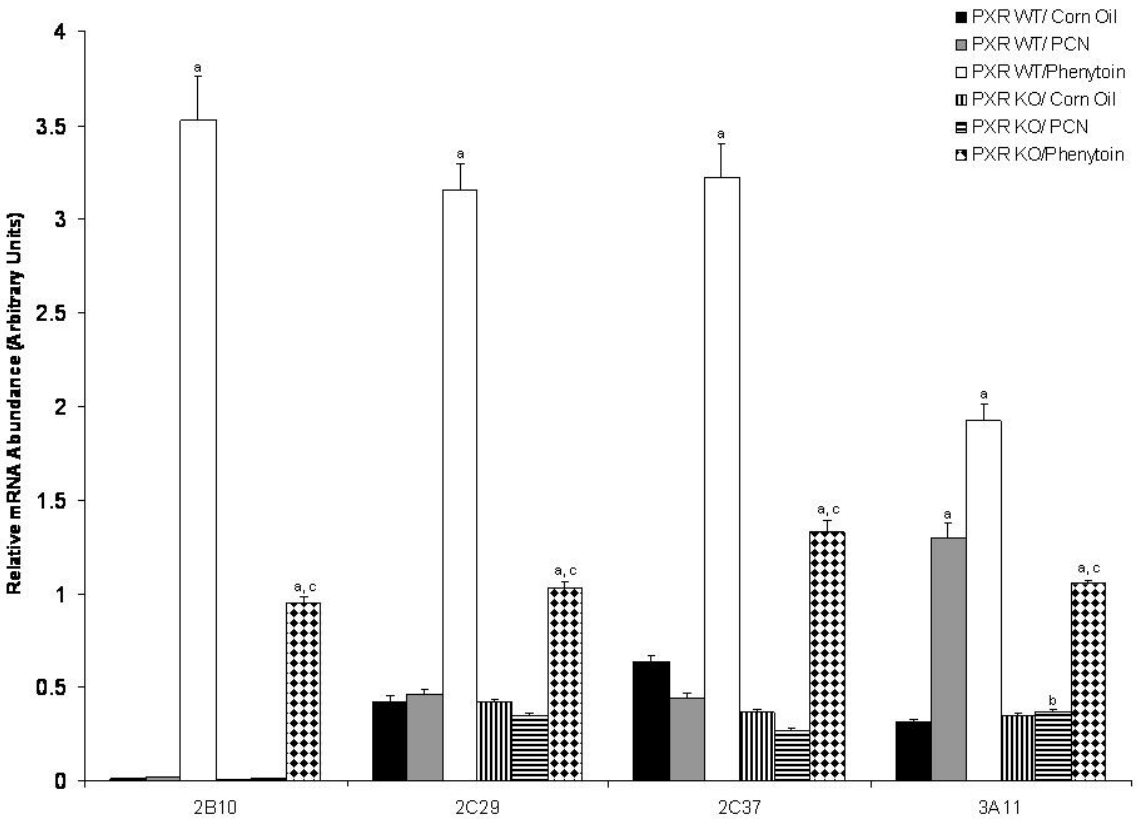

Figure 3.8- Drug response of hepatic CYP2B10, CYP2C29, CYP2C37, and CYP3A11 mRNA in SV129/C57B6 wild-type and PXR-null mice. Mice were treated as described previously. Mice treated with PCN (80 mg/kg) were treated orally for three consecutive days. Quantitative RT-PCR was performed to evaluate target gene mRNA content in response to PCN and phenytoin. Target genes were normalized to a reference gene, β-actin. CYP2B10, CYP2C29, and CYP2C37 mRNA was induced in wild-type mice ~346 fold, ~7 fold, and ~5 fold by phenytoin respectively. In PXR-null mice, CYP2B10, CYP2C29, and CYP2C37 mRNA induction by phenytoin was reduced ~74%, ~68%, and ~60%, respectively. CYP3A11 mRNA is induced ~4 fold by PCN in wild-type mice, but was abolished in PXR-null mice. CYP3A11 mRNA was induced by phenytoin ~6 fold in wild-type mice, but was reduced ~45% in PXR-null mice. Values expressed above represent the relative abundance of gene specific mRNA +/- SE. P-values were determined using a LSMeans pairwise contrast comparison with a Bonferroni correction. *[a] p< 0.01, significantly higher than corn oil controls. [b] p < 0.01, significantly lower than wild-type treated with PCN. [c] p < 0.01, significantly lower than wild-type treated with phenytoin.*

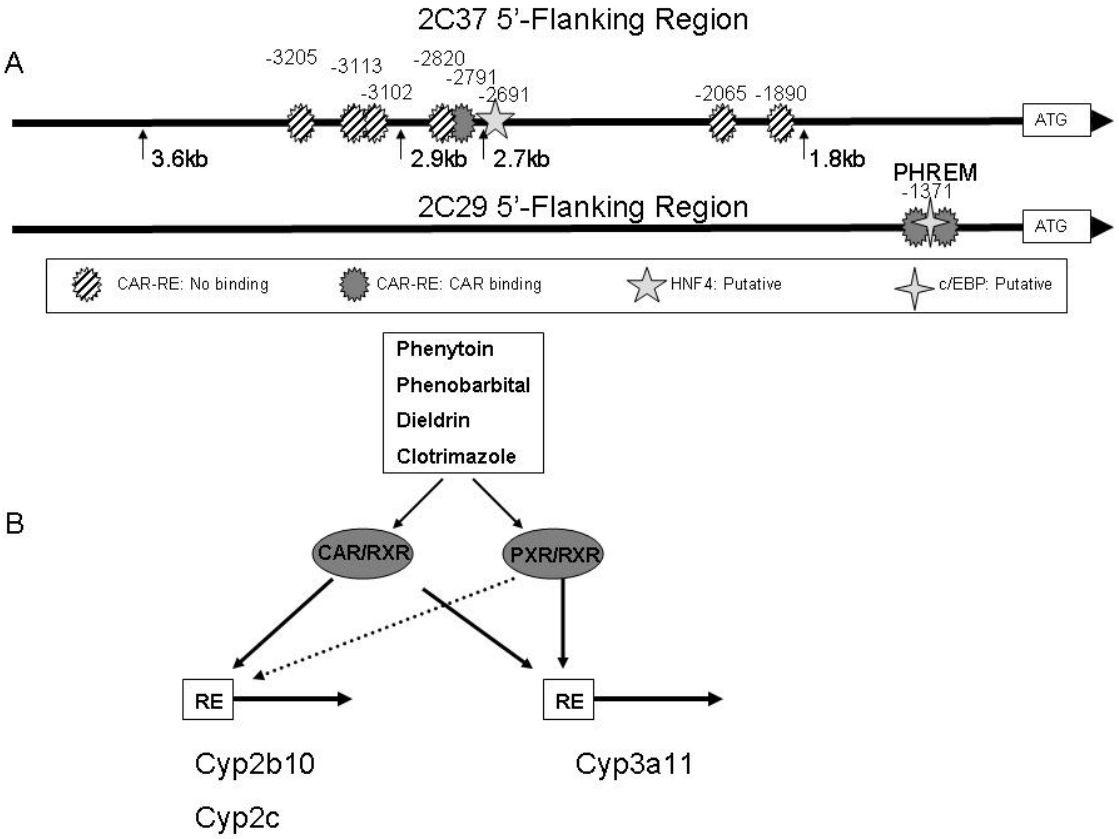

Figure 3.9- Summary illustration and comparison of *Cyp2c37* and *Cyp2c29* 5'-flanking regions. A: A schematic representation of the *Cyp2c37* and the *Cyp2c29* 5'-flanking regions providing a summary of putative and functional transcription factor binding sites. B: Illustration showing the shared activators of the xenobiotic sensing nuclear receptors PXR and CAR and their respective genes that are induced via shared response elements.

4.0 Conclusions

The commonly used antiepileptic drug phenytoin has long been associated with drug-drug interactions presumably resulting from the induction of drug metabolizing enzymes such as the P450 enzymes. Phenytoin has been shown to induce the P450 subfamilies CYP3A, CYP2B, and the CYP2C (Raucy, 2003; Wang et al., 2003b; Ferguson et al., 2005). Drug induction of P450 enzymes often results in drug-drug interactions leading to altered drug efficacy and or toxicity. These interactions are of considerable concern and are common in patients subjected to combination drug therapy such as those suffering from epilepsy.

The CYP2C subfamily plays a significant role in the metabolism of several clinical drugs including phenytoin (Goldstein and de Morais, 1994; Goldstein and Blaisdell, 1996; Goldstein, 2001). Induction of the P450 genes is frequently regulated at the transcriptional level and is usually mediated by the xenobiotic nuclear receptors CAR and PXR (Honkakoski et al., 1998b; Goodwin et al., 1999; Ferguson et al., 2002; Goodwin, 2002; Goodwin et al., 2002; Chen et al., 2003; Wang et al., 2003a; Chen et al., 2004; Ferguson et al., 2005). Substrates such as phenytoin are often activators of CAR or PXR, thus inducing the enzymes that mediate their metabolism. However, no conclusive evidence has indicated whether PXR or CAR mediates phenytoin induction, thus the murine model provided an excellent system to investigate the molecular mechanisms regulating CYP2C induction by this commonly used antiepileptic. The studies presented herein identified: 1) two phenytoin and phenobarbital inducible murine CYP2C genes, *Cyp2c29* and *Cyp2c37*, 2) functional nuclear receptor response elements located

within the 5'-flanking regions of these genes, and 3) the nuclear receptor responsible for mediating phenytoin induction of *Cyp2c29*, *Cyp2c37*, *Cyp2b10*, and *Cyp3a11* using the CAR-null and PXR-null mice strains.

The studies in Chapters 2-3 identified two inducible murine CYP2C genes, *Cyp2c29* and *Cyp2c37*, by phenobarbital and phenytoin. Due to the overwhelming similarity in the coding sequences of the murine CYP2C genes, quantitative RT-PCR was required to identify specific CYP2C isoforms. These studies demonstrated that CYP2C29 and CYP2C37 mRNA was increased by phenobarbital and phenytoin treatment. These studies also showed that the increase in mRNA by phenytoin was accompanied with a concomitant increase in CYP2C29 and CYP2C37 protein as indicated by immunblots. These proteins were identified using polyclonal antibodies raised against specific peptide sequences derived from CYP2C29 or CYP2C37 amino acid sequence. The increase in mRNA and protein suggested that the induction of these genes by phenytoin was regulated on the transcriptional level. These findings were consistent with phenobarbital induction of the human genes *CYP3A4*, *CYP2B6, CYP2C8*, and *CYP2C9* and the mouse gene *Cyp2b10* (Honkakoski et al., 1998a; Honkakoski et al., 1998b; Sueyoshi et al., 1999; Gerbal-Chaloin et al., 2001; Ferguson et al., 2002; Goodwin, 2002; Raucy et al., 2002; Wang et al., 2003b; Chen et al., 2004; Ferguson et al., 2005).

CAR and PXR have been shown to increase transcription in response to drugs by binding response elements located within the 5'-flanking regions of target genes (Honkakoski et al., 1998b; Goodwin et al., 1999; Sueyoshi et al., 1999; Ferguson et al., 2002; Goodwin, 2002; Goodwin et al., 2002; Chen et al., 2003;

Wang et al., 2003a; Chen et al., 2004; Ferguson et al., 2005). In chapter 2, a novel PHREM was identified within the 5'-flanking region of *Cyp2c29* located at ~1.3 kb from the translational start site. The PHREM consisting of two imperfect DR-4 motifs separated by 17 nucleotides and was very similar to the PBREM identified in the 5'-flanking regions of both *Cyp2b10* and *CYP2B6*. Mutagenesis of both DR-4 elements of the PHREM abolished induction in phenytoin and phenobarbital treated mice indicating that the PHREM is necessary for induction of *Cyp2c29* by phenytoin and phenobarbital. These findings were consistent with similar studies of the PBREM removing phenobarbital induction of the *Cyp2b10* gene. In contrast, only one active CAR-RE was identified within the 5'-flanking sequence of the murine *Cyp2c37* gene. Mutagenesis studies in chapter 3 demonstrated that this imperfect DR-4 was the principle CAR responsive element within -2.9 kb of *Cyp2c37* translation start site. Similarly, only one functional CAR-RE was identified as the principle element conferring CAR responsiveness in the human genes *CYP2C8* and *CYP2C19* (Chen et al., 2003; Ferguson et al., 2005). Not surprisingly, *Cyp2c37* luciferase reporters were not transactivated by mCAR in HepG2 cells as well as *Cyp2c29* luciferase reporters. The presence of an additional active CAR-RE within the 5'-flanking sequence of *Cyp2c29* may explain this observed difference. This hypothesis is consistent with the identification of additional active nuclear response elements essential for maximum induced expression of *CYP3A4* and *CYP2B6* (Goodwin et al., 1999; Wang et al., 2003a).

In recent studies, phenytoin has been shown to induce human *CYP3A4* and *CYP2B6*, but the low PXR-mediated increase in *CYP3A4* and CYP2B6 promoter

luciferase activity was inconsistent with the high inducibility of the mRNA (Raucy, 2003; Wang et al., 2003b). These studies lacked conclusive evidence to indicate whether PXR or CAR mediated phenytoin induction. In Chapters 2 and 3, we examined the induction of CYP2C29, CYP2C37, CYP2B10, and CYP3A11 mRNA by phenytoin in CAR-null and PXR-null mice to determine whether CAR or PXR mediated phenytoin induction. In CAR-null mice, the induction of CYP2B10 mRNA by phenytoin and phenobarbital was completely removed. Similarly, the induction of CYP2C29 and CYP2C37 mRNA by phenytoin and phenobarbital was dramatically reduced in CAR-null mice. However in PXR-null mice, phenytoin induction of CYP2B10, CYP2C29, and CYP2C37 mRNA were only moderately reduced. Taken together, these results indicated that the induction of the *Cyp2b10*, *Cyp2c29*, and *Cyp2c37* genes by phenytoin and phenobarbital is CAR-dependent.

The induction of the *Cyp2b10* gene by phenobarbital has been shown to be regulated by CAR (Honkakoski et al., 1998a; Honkakoski et al., 1998b). Phenobarbital is not believed to be a ligand of CAR, but instead induces the *Cyp2b10* gene by initiating the nuclear translocation of CAR via a presently unidentified signaling pathway (Sueyoshi and Negishi, 2001). In contrast, TCPOBOP is a mCAR ligand that inhibits the repression of mCAR by androstenol (Forman et al., 1998; Tzameli et al., 2000). Neither phenytoin nor phenobarbital inhibited androstenol repression of mCAR in transiently cotransfected HepG2 cells. These results suggest that phenytoin is probably not a mCAR ligand. However, immunoblot analysis of hepatic nuclear extracts indicated that phenytoin induces

nuclear translocation of mCAR, thereby inducing the genes *Cyp2b10*, *Cyp2c29*, and *Cyp2c37* via a mechanism similar to phenobarbital.

Although phenytoin appears to be a pure CAR agonist in the induction of the genes *Cyp2b10*, *Cyp2c29*, and *Cyp2c37*, it is not clear whether phenytoin induces all P450 genes via CAR. The compounds dieldrin and clotrimazole have been shown to induce both *Cyp2b10* and *Cyp3a11* in wild-type mice (Wei et al., 2002; Zhang et al., 2004). In CAR-null mice, induction of CYP2B10 mRNA by either compound is abolished; clearly demonstrating that CYP2B10 induction is mediated by CAR (Wei et al., 2002; Zhang et al., 2004). However, induction of CYP3A11 mRNA by dieldrin or clotrimazole is apparently mediated by both receptors since it is only abolished in CAR/PXR double null mice (Zhang et al., 2004). Similarly, the induction of murine CYP3A11 mRNA by phenytoin was not abolished, but only moderately reduced in both single null mice strains. These results suggest that the induction of *Cyp3a11* gene by phenytoin may also be mediated by both receptors; therefore, phenytoin is a mixed CAR/PXR agonist similar to dieldrin and clotrimazole. In contrast, the induction of CYP3A11 mRNA by PCN was completely removed in PXR-null mice consistent with PXR mediated induction (Xie et al., 2000; Staudinger et al., 2001a; Staudinger et al., 2001b; Goodwin et al., 2002). Furthermore, PCN did not induce CYP2B10, CYP2C29, and CYP2C37 mRNA in wild-type mice suggesting that these genes are predominately regulated by CAR. Surprisingly, the constitutive expression of CYP2B10, CYP2C29, and CYP2C37 mRNA was significantly reduced in CAR-null mice, but not in PXR-null mice. These

results may indicate that CAR plays a larger role in regulating constitutive expression than PXR.

In summary, these studies have identified two phenobarbital and phenytoin inducible murine CYP2C genes, *Cyp2c29* and *Cyp2c37*. We identified and characterized a novel CAR-RE, PHREM, located ~1.3 kb from *Cyp2c29* translational start site. This site was shown to be essential for the induction of *Cyp2c29* by phenytoin and phenobarbital. We also identified and characterized a functional CAR-RE located ~1.8 kb from the translational start site of *Cyp2c37*. This site was shown to be necessary for mCAR transactivation of -2.9 kb *Cyp2c37* luciferase reporter. In addition, phenytoin was demonstrated to elicit the nuclear accumulation of CAR indicating that phenytoin is an agonist of CAR similar to phenobarbital. Quantitative RT-PCR in CAR-null and PXR-null mice established that phenytoin is a shared agonist of CAR and PXR similar to dieldrin and clotrimazole. These results also indicated that the induction of the *Cyp2b10*, *Cyp2c29*, *Cyp2c37*, and *Cyp3a11* genes by phenytoin is mediated in a gene promoter dependent manner. Furthermore, quantitative RT-PCR revealed that the constitutive expression of CYP2B10, CYP2C29, and CYP2C37 mRNA was significantly lower in CAR-null mice, but remained unchanged in PXR-null mice. These results clearly establish a direct or indirect role for CAR in the constitutive regulation of these genes and suggest that PXR is primarily involved in drug induction. These results are consistent with studies showing that CAR is a multifaceted transcription factor (Ueda et al., 2002).

4.1 References Cited

Chen Y, Ferguson SS, Negishi M and Goldstein JA (2003) Identification of constitutive androstane receptor and glucocorticoid receptor binding sites in the CYP2C19 promoter. *Mol Pharmacol* 64:316-324.

Chen Y, Ferguson SS, Negishi M and Goldstein JA (2004) Induction of human CYP2C9 by rifampicin, hyperforin, and phenobarbital is mediated by the pregnane X receptor. *J Pharmacol Exp Ther* 308:495-501.

Ferguson SS, Chen Y, LeCluyse EL, Negishi M and Goldstein JA (2005) Human CYP2C8 is transcriptionally regulated by the nuclear receptors constitutive androstane receptor, pregnane X receptor, glucocorticoid receptor, and hepatic nuclear factor 4alpha. *Mol Pharmacol* 68:747-757.

Ferguson SS, LeCluyse EL, Negishi M and Goldstein JA (2002) Regulation of human CYP2C9 by the constitutive androstane receptor: discovery of a new distal binding site. *Mol Pharmacol* 62:737-746.

Forman BM, Tzameli I, Choi HS, Chen J, Simha D, Seol W, Evans RM and Moore DD (1998) Androstane metabolites bind to and deactivate the nuclear receptor CAR-beta. *Nature* 395:612-615.

Gerbal-Chaloin S, Pascussi JM, Pichard-Garcia L, Daujat M, Waechter F, Fabre JM, Carrere N and Maurel P (2001) Induction of CYP2C genes in human hepatocytes in primary culture. *Drug Metab Dispos* 29:242-251.

Goldstein JA (2001) Clinical relevance of genetic polymorphisms in the human CYP2C subfamily. *Br J Clin Pharmacol* 52:349-355.

Goldstein JA and Blaisdell J (1996) Genetic tests which identify the principal defects in CYP2C19 responsible for the polymorphism in mephenytoin metabolism. *Methods Enzymol* 272:210-218.

Goldstein JA and de Morais SM (1994) Biochemistry and molecular biology of the human CYP2C subfamily. *Pharmacogenetics* 4:285-299.

Goodwin B, Hodgson E and Liddle C (1999) The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. *Mol Pharmacol* 56:1329-1339.

Goodwin B, Hodgson, E., D"Costa, D.J., Robertson, G.R., Liddle, C. (2002) Transcriptional Regulation of the Human *CYP3A4* Gene by the Constitutive Androstane Receptor. *Molecular Pharmacology* 62:359-365.

Goodwin B, Redinbo MR and Kliewer SA (2002) Regulation of cyp3a gene transcription by the pregnane x receptor. *Annu Rev Pharmacol Toxicol* 42:1-23.

Honkakoski P, Moore R, Washburn KA and Negishi M (1998a) Activation by diverse xenochemicals of the 51-base pair phenobarbital-responsive enhancer module in the CYP2B10 gene. *Mol Pharmacol* 53:597-601.

Honkakoski P, Zelko I, Sueyoshi T and Negishi M (1998b) The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the CYP2B gene. *Mol Cell Biol* 18:5652-5658.

Raucy JL (2003) Regulation of CYP3A4 expression in human hepatocytes by pharmaceuticals and natural products. *Drug Metab Dispos* 31:533-539.

Raucy JL, Mueller L, Duan K, Allen SW, Strom S and Lasker JM (2002) Expression and induction of CYP2C P450 enzymes in primary cultures of human hepatocytes. *J Pharmacol Exp Ther* 302:475-482.

Staudinger J, Liu Y, Madan A, Habeebu S and Klaassen CD (2001a) Coordinate regulation of xenobiotic and bile acid homeostasis by pregnane X receptor. *Drug Metab Dispos* 29:1467-1472.

Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, Reinhard J, Willson TM, Koller BH and Kliewer SA (2001b) The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc Natl Acad Sci U S A* 98:3369-3374.

Sueyoshi T, Kawamoto T, Zelko I, Honkakoski P and Negishi M (1999) The repressed nuclear receptor CAR responds to phenobarbital in activating the human CYP2B6 gene. *J Biol Chem* 274:6043-6046.

Sueyoshi T and Negishi M (2001) Phenobarbital response elements of cytochrome P450 genes and nuclear receptors. *Annu Rev Pharmacol Toxicol* 41:123-143.

Tzameli I, Pissios P, Schuetz EG and Moore DD (2000) The xenobiotic compound 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene is an agonist ligand for the nuclear receptor CAR. *Mol Cell Biol* 20:2951-2958.

Ueda A, Hamadeh HK, Webb HK, Yamamoto Y, Sueyoshi T, Afshari CA, Lehmann JM and Negishi M (2002) Diverse roles of the nuclear orphan receptor CAR in regulating hepatic genes in response to phenobarbital. *Mol Pharmacol* 61:1-6.

Wang H, Faucette S, Sueyoshi T, Moore R, Ferguson S, Negishi M and LeCluyse EL (2003a) A novel distal enhancer module regulated by pregnane X receptor/constitutive androstane receptor is essential for the maximal induction of CYP2B6 gene expression. *J Biol Chem* 278:14146-14152.

Wang H, Faucette SR, Gilbert D, Jolley SL, Sueyoshi T, Negishi M and LeCluyse EL (2003b) Glucocorticoid receptor enhancement of pregnane X receptor-mediated CYP2B6 regulation in primary human hepatocytes. *Drug Metab Dispos* 31:620-630.

Wei P, Zhang J, Dowhan DH, Han Y and Moore DD (2002) Specific and overlapping functions of the nuclear hormone receptors CAR and PXR in xenobiotic response. *Pharmacogenomics J* 2:117-126.

Xie W, Barwick JL, Downes M, Blumberg B, Simon CM, Nelson MC, Neuschwander-Tetri BA, Brunt EM, Guzelian PS and Evans RM (2000) Humanized xenobiotic response in mice expressing nuclear receptor SXR. *Nature* 406:435-439.

Zhang J, Huang W, Qatanani M, Evans RM and Moore DD (2004) The constitutive androstane receptor and pregnane X receptor function coordinately to prevent bile acid-induced hepatotoxicity. *J Biol Chem* 279:49517-49522.