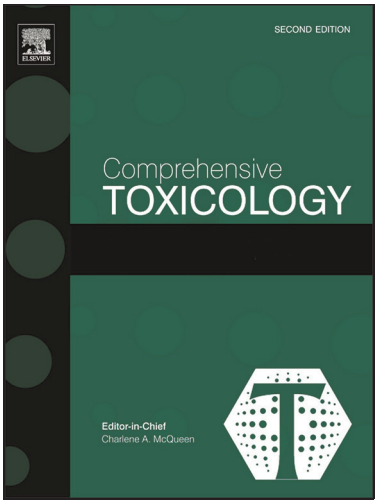

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2.10 Constitutive Androstane Receptor

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Abbreviations

AF1	activation function 1
AhR	aryl hydrocarbon receptor
BTE	basal transcription element
CAR	constitutive androstane receptor
CCRP	cytoplasmic CAR retention protein
CYP	cytochrome P450
DNA	deoxyribonucleic acid
FXR	farnesoid X receptor
GRE	glucocorticoid response element
LXR	liver X receptor
mRNA	messenger ribonucleic acid
NF-1	nuclear factor-1
PB	phenobarbital
PP2A	protein phosphatase 2A
PPARα	peroxisome proliferator-activated receptor α
PXR	pregnane X receptor
RAR	retinoic acid receptor
RXR	retinoid X receptor
THR	thyroid hormone receptor
VDR	vitamin D receptor
XRS	xenochemical response signal

2.10.1 Introduction

In mammalian organisms, during development and under basal conditions, a repertoire of approximately 25 000 genes (International Human Genome Sequencing Consortium 2004) undergoes differential transcription. Activation of specific genes in response to chemicals, pathogenic infection, or environmental stressors requires a highly integrated signal transduction process that signals the transcriptional machinery to direct the expression patterns of appropriate genes (Lemon and Tjian 2000). The activation of a given gene will depend on the simultaneous interplay of particular combinations of nuclear proteins that localize to their promoter regions and other regulatory deoxyribonucleic acid (DNA) elements, such as transcriptional enhancers. Most DNA enhancer elements contain distinct sets of transcription factor binding sites. Variation in the arrangement of these sites provides the potential to create unique and context-specific DNA–protein complexes (Carey 1998; Kim and Maniatis 1997). Cooperative interplay between the proteins in these complexes and with other nuclear factors, such as coactivators and corepressors, can lead to a high level of discrimination in gene activation and to a marked level of transcription synergy (Carey 1998; Lemon and Tjian 2000; Lin *et al.* 1990).

It is noteworthy that several classes of environmental and therapeutic substances are recognized for their capacity to markedly modulate the transcriptional status of mammalian biotransformation enzymes. These enzymes include certain glutathione-*S*-epoxide transferases, UDP-glucuronosyl

transferases, epoxide hydrolases, aldehyde dehydrogenases, and the cytochrome P450s (CYPs) (Denison and Whitlock 1995; Fuhr 2000; Gonzalez 1988; Kemper 1998; Sueyoshi and Negishi 2001). The CYPs constitute a very important phase I enzyme network and principally catalyze the oxidization of a wide variety of chemicals, including pharmaceuticals. Typically, the biotransformation process tends toward detoxification, with the resulting metabolites being more water-soluble and exhibiting increased likelihood to undergo further reactions via phase II conjugation pathways. However, a large number of procarcinogens and other environmental toxins are bioactivated by the xenobiotic-metabolizing CYPs (Guengerich 2000).

The CYP monooxygenases are believed to have evolved from an ancestral gene 3.5–4.0 billion years ago (Nelson 1999). The CYPs are intimately involved in functionalization reactions representing the first phase of xenobiotic detoxification, as well as being integral parts of many biosynthetic reactions in the cell. Over 1000 CYP genes have been characterized across many species of animals, plants, and microbes (Nelson 1999). It has been proposed that evolution of more recent CYP genes coincided with terrestrial colonization of plant-eating animals 400 million years ago. One theory proposes that an animal–plant war began as plants synthesized toxic compounds to discourage predators, and animals responded to this selective pressure by evolving multiple CYP genes to detoxify the novel toxins (Nebert and McKinnon 1994; Nelson 1999). Different CYP family members tend to exhibit substrate preferences; however, extensive overlap in substrate specificity does exist (Nelson *et al.* 1996). CYPs are expressed in most mammalian tissues; however, the liver is responsible for the bulk of chemical biotransformation (Fuhr 2000; Omiecinski *et al.* 1999).

Since many relevant substances are either bioactivated or detoxified by CYP-mediated metabolism, it is likely that variation in expression patterns and levels of CYPs broadly impact the outcome of chemical exposures (Eaton 2000). It is further anticipated that certain interindividual differences in CYP expression may lead to altered risk for the development of toxicities, such as certain cancers, birth defects, and adverse drug reactions (Eaton 2000; Guengerich 2000; Nebert 1997). This chapter will focus on how these systems are regulated by the constitutive androstane receptor (CAR; NR1I3) with specific emphasis on its role in the phenobarbital (PB) induction response, the phenomenon whereby PB and PB-like agents modulate expression of the mammalian biotransformation system. This topic has been the subject of several reviews, to which the reader is directed for further information (Kemper 1998; Kodama and Negishi 2006; Sueyoshi and Negishi 2001; Waxman 1999).

2.10.2 The PB Induction Response

There are several prototypical inducing agents, including the polyaromatic and polychlorinated hydrocarbons, ethanol and organic solvents, peroxisome proliferator compounds such as the phthalate esters, dexamethasone, and several sedative-hypnotic medications (Denison and Whitlock 1995; Fuhr 2000; Kemper 1998). These different classes of inducers tend to impact the expression levels of the CYP1A, CYP2E, CYP4A, CYP3A, and CYP2B subfamilies of P-450, respectively. In the last case, PB serves as a model agent for other barbiturates and a variety of xenobiotic compounds such as chlordane, dichlorodiphenyltrichloroethane (DDT), and certain PCBs that exhibit profound inductive effects on the biotransformation system (Kemper 1998; Waxman and Azaroff 1992). The PB induction response occurs in most mammalian species, including humans, and is principally manifested in the liver (Fuhr 2000). Details of the other processes that relate to the various inducer pathways are the subject of other chapters in this volume.

Individuals can differ in the relative levels of CYPs that are expressed constitutively; similarly, a number of members of these principal CYP subfamilies are markedly inducible upon exposure to chemicals. For example, in rats, PB treatments can induce both CYP2B1 and CYP2B2 levels in liver up to 50- to 100-fold (Omiecinski 1986; Omiecinski *et al.* 1992). PB-inducible responses have been documented for human CYP2B6 in primary hepatocyte culture (Gervot *et al.* 1999; Olsavsky *et al.* 2007; Page *et al.* 2007a). The human and rodent CYP3A4 genes are similarly markedly responsive to prototypical PB-like inducers (Hassett *et al.* 1998; Sidhu and Omiecinski 1995). CYP gene induction can manifest both beneficial and detrimental effects on xenobiotic metabolism. Understanding the mechanisms that account for these induction responses may greatly facilitate both therapeutic and prophylactic intervention in disease states.

Much of the work on the PB induction response has focused on the CYP2B family. The CYP2B genes

are typically expressed in the liver at very low basal levels. Similarly, low levels of CYP2B messenger ribonucleic acid (mRNA) can be detected in selected other tissues (Lake *et al.* 1993; Omiecinski 1986). When an inducer is present, mRNA and protein levels increase markedly, up to 100-fold over basal levels. The induction response is relatively liver specific, although reports indicate that the PB induction response is also manifested in intestinal enterocytes and in the brain (Schilter *et al.* 2000; Traber *et al.* 1988). However, in the latter tissues, the induced levels of expression are far lower than those occurring in the liver. Within the liver, inducible CYP2B expression is most pronounced in the centrilobular region, that is, those liver hepatocytes in closest proximity to the central vein (Oinonen and Lindros 1998). The underlying mechanisms controlling tissue- and regiospecific expression and induction responses remain relatively poorly understood. Through study of the rodent PB-inducible P-450 genes, in particular the mouse Cyp2b10 and the rat CYP2B1 and CYP2B2, a great deal of progress has been made regarding the molecular mechanisms involved in the PB induction response. The principal features of the 5′-flanking promoter regions of these genes, as well as the orthologous human CYP2B6 gene, appear quite similar (Honkakoski and Negishi 1998; Sueyoshi and Negishi 2001). The hallmark structural features are described below and indicated in **Figure 1**.

The CYP2B genes contain the typical DNA elements (*cis* elements) that serve as binding sites for transcription factors (*trans* elements) found in many eukaryotic genes (see **Figure 1**). The respective CYP2B promoter contains *cis* elements, such as the TATAA box, and other elements required for RNA polymerase complex formation. This region is referred to as the proximal promoter region and is involved in directing the basal transcriptional levels of the gene (Sommer *et al.* 1996). The proximal region is just upstream of the transcription start site, where the structural DNA begins its transcription into heterogeneous RNA for subsequent splicing into mature mRNA.

Initially, experiments conducted in the PB-inducible bacteria *Bacillus megaterium* revealed the existence of a 17 bp operon termed the Barbie box (He and Fulco 1991) that responded to PB by increasing the transcription of CYP102 and CYP106 genes (Narhi and Fulco 1982). This response is mediated by both positive- and negative-acting transcription factors that bind to a *cis* element (Liang *et al.* 1995) on the operon. Although putative Barbie box consensus elements were subsequently identified in several mammalian PB-responsive genes, including certain CYP genes (He and Fulco 1991), further investigations demonstrated that the Barbie box does not appear to alter PB-mediated induction of CYP genes in eukaryotes (Kemper 1998; Palmer *et al.* 1999; Shaw *et al.* 1998).

A basal transcription element (BTE) was also studied in the proximal region of the promoter in several PB-inducible CYP genes (Foti *et al.* 1998; Park and Kemper 1996). Both the BTE and the Barbie box elements may be involved in basal transcriptional regulation, but are not likely critical in directing PB induction (Liu *et al.* 1998). Additional DNA elements exist further upstream of the proximal promoter region of the CYP2B1/2 and Cyp2b10 genes that appear to interact *in vitro* with a variety of transcription factors. For example, a functional CCAAT/enhancer binding protein β (C/EBPα) response element was reported as important for the constitutive transcription in the CYP2B1 gene (Luc *et al.* 1996; Park and Kemper 1996). Further upstream, nuclear factor κB (NF-κB) sites were identified in the CYP2B gene promoters from rat, mouse, and human that may be involved in gene repression

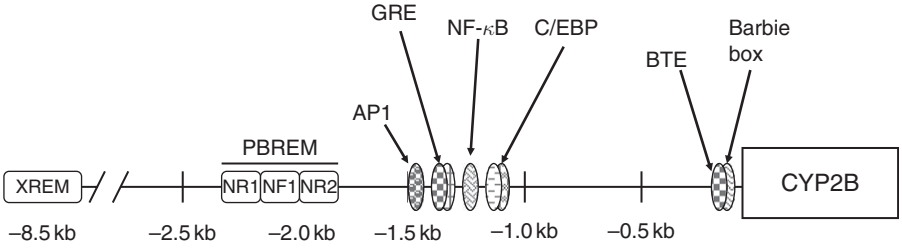

Figure 1 Structure of the upstream regulatory region for a generic CYP2B gene. The relative positions of the phenobarbital response enhancer module (PBREM) and the xenobiotic responsive enhancer module (XREM) are shown as well as other potential regulatory sites and the core promoter region of the gene. The PBREM and XREM are believed to be the most important regulatory components for the phenobarbital induction response in mammals.

(Lee *et al.* 2000). This potential mode of regulation may be important since a number of inflammatory processes, cytokines, and viral exposures have been reported to repress the PB induction process (Morgan 1997). A glucocorticoid response element (GRE) was characterized in the CYP2B2 promoter approximately 1.3 kb 5′ of the transcription start site (Jaiswal *et al.* 1990) that may be involved in the apparent glucocorticoid dependence of the PB induction response (Schuetz *et al.* 2000; Shaw *et al.* 1993; Sidhu and Omiecinski 1995). Other transcription factors, such as activator protein-1 (AP1), may also be involved in regulating the CYP2B genes (Roe *et al.* 1996).

Transgenic studies in mice were the first to implicate the existence of the upstream PB enhancer region, using rat CYP2B2 promoter constructs as mouse 'transgenes' (Ramsden *et al.* 1993). Using both primary hepatocyte culture models (Honkakoski *et al.* 1996, 1998; Trottier *et al.* 1995) and direct *in situ* injection of DNA into the liver (Park *et al.* 1996), investigators successfully delineated a 51 bp PBREM approximately 2.3 kb upstream of the core promoters of the rat CYP2B2, mouse Cyp2b10, and human CYP2B6 (Sueyoshi *et al.* 1999) PB-inducible genes. The Negishi group was the first to identify the interaction of the CAR (NR1I3) within the PBREM region of the mouse Cyp2b10 gene (Honkakoski *et al.* 1998). The PBREM appears to be intimately involved in the induction or upregulation response subsequent to exposure to the PB class of agents (Honkakoski and Negishi 1998; Trottier *et al.* 1995). By definition, an enhancer fragment of DNA can function in either orientation. In this respect, it is noteworthy that the PBREM region is in the opposite orientation in the human CYP2B6 gene compared to that of the corresponding rat or mouse genes (Sueyoshi *et al.* 1999). With the discovery of the enhancer region, the anatomy of the enhancer and its regulatory factor interactions have been studied in detail.

As indicated in **Figure 1**, the PBREM is composed principally of two nuclear receptor sites, NR1 and NR2. These sites are termed 'NR' because the response element exhibits an imperfect direct repeat nucleotide sequence known to bind nuclear receptors. The NR sites within the PBREM flank a core nuclear factor-1 (NF-1) motif (Liu *et al.* 1998). NF-1 is a liver-enriched nuclear factor involved in the regulation of many genes expressed in the liver. The potential role of the NF-1 element in the PB induction process has been examined in various models. The NF-1 region is marked by a strong DNase I footprint, and coincides with a DNase I hypersensitivity region (Liu *et al.* 1998). Although NF-1 itself does not appear to possess PB-mediated transactivation activity in transfected cells, mutation of the NF-1 site within CYP2B genes reduced the PB induction response in *in vitro* transfection assays (Honkakoski *et al.* 1998; Liu *et al.* 1998; Stoltz *et al.* 1998). Studies of NF-1 interaction on chromatinized templates indicate that NF-1 binds independent of other nuclear factors to the PBREM but may enhance the nuclear factor activity during the induction response (Kim *et al.* 2000). However, results from studies using CYP2B2 transgenic mouse models that contained loss-of-function mutations in the NF-1 motif indicated no substantial loss of PB inducibility with the mutant NF-1 transgenes, arguing against a critical role of the factor in directing the PB induction response *in vivo* (Ramsden *et al.* 1999). Interestingly, interactions of other transcriptional proteins as accessory factors within the PBREM domain have also been reported. These potentially interacting factors include the glucocorticoid receptor (Stoltz *et al.* 1998), HNF-4 (Beaudet *et al.* 2005), and the liver X receptor (LXR) (Beaudet *et al.* 2005), interactions that may allow for a complex interplay of regulators affecting PBREM transcriptional functionality. Furthermore, a novel distal enhancer module (XREM; see **Figure 1**) that is regulated by pregnane X receptor (PXR)/CAR has been identified far upstream of the PBREM in the human CYP2B6 gene and it appears to be essential for maximal induction of CYP2B6 expression (Wang *et al.* 2003). Several other mechanisms may be operative in the induction process that occurs in response to the PB-class of agents, including posttranscriptional stabilization of mRNA, posttranslational stabilization of the enzyme, posttranslational protein modifications such as changes in phosphorylation status, and/or direct transcriptional activation of the respective genes. Together, these observations point to a dynamic integration of the PB induction response in mammalian liver.

Although perhaps complex on the one hand, it does appear that a principal pathway directing the PB-inducible response in hepatocytes involves direct gene activation by CAR at the transcriptional level (Hardwick *et al.* 1983). Therefore, the remainder of this chapter will focus on CAR as a relatively newly discovered transcription factor that preferentially activates the PBREM and a factor that has also been implicated in a number of other areas of liver physiology.

2.10.3 The Constitutive Androstane Receptor

2.10.3.1 Initial Cloning and Characterization

Originally cloned from human liver as MB67 (Baes *et al.* 1994) and later from mouse (Choi *et al.* 1997), CAR is a member of the nuclear receptor superfamily that is expressed primarily in the liver. CAR was determined to constitutively activate target genes by binding direct repeat (DR-4 and DR-5) elements as a heterodimer with the retinoid X receptor (RXR) (Baes *et al.* 1994; Choi *et al.* 1997). Other DNA binding motifs have also been identified for CAR, including DR-1s, DR-3s, ER-8s, and IR-2s (Echchgadda *et al.* 2007; Frank *et al.* 2003). In the adult liver, RXRα is the most abundant of the three RXR receptors, suggesting an important role as a regulator of gene expression in this organ (Mangelsdorf *et al.* 1992). The importance of RXRα is quite clear in a broader biological context in that it engages as a dimer partner with a large number of other nuclear receptors, including the retinoic acid receptors (RARs), vitamin D receptor (VDR), thyroid hormone receptor (THR), peroxisome proliferator-activated receptor α (PPARα), PXR, LXR, and the farnesoid X receptor (FXR) (Mangelsdorf and Evans 1995; Waxman 1999). In this context, RXR has been referred to as a master regulator (Mangelsdorf and Evans 1995).

As noted earlier, the Negishi group was the first to identify the interaction of CAR within the PBREM region of the mouse Cyp2b10 gene (Honkakoski *et al.* 1998). Further studies showed that CAR also activated the human orthologue of Cyp2b10, CYP2B6, as well as the human CYP3A4 gene (Sueyoshi *et al.* 1999), indicating a combinatorial regulation of CYP3A4 by both CAR and the PXR. PXR was later shown to also regulate the human CYP2B6 gene (Goodwin *et al.* 2001), indicative of receptor promiscuity, or functional overlap in certain cases. It was also shown that activation of CAR occurs through a nuclear translocation mechanism after treatment with PB. In untreated mice, CAR is sequestered in the cytoplasm of the hepatocytes, but upon treatment with PB, CAR accumulates in the nucleus and activates target genes (Kawamoto *et al.* 1999). Together these results indicate that CAR is a major transcription factor involved in the PB induction response. This conclusion has been further verified by generation of a CAR knockout mouse model that exhibits none of the induction responses typically seen with PB exposure (Wei *et al.* 2000).

2.10.3.2 Regulation of Transcription

In stably transfected HepG2 cells and transiently transfected cell lines, CAR displayed ligand-independent activation of PBREM reporter genes, confirming the initial reports of constitutive activity (Honkakoski *et al.* 1998) (it is sometimes referred to as the 'constitutively active receptor'). This constitutive nature of CAR requires mechanisms of regulation apart from ligand binding. *In vivo*, CAR resides mostly in the cytoplasm, sequestered there by a mechanism that is not yet fully understood, although it is likely that it is retained in a protein complex with hsp90 (Yoshinari *et al.* 2003), the cytoplasmic CAR retention protein (CCRP) (Kobayashi *et al.* 2003), and PPP1R16A (Sueyoshi *et al.* 2008). Activation of CAR occurs when it translocates to the nucleus and elicits target gene expression, a process that has been shown to occur after treatment with PB or PB-like inducers (Kawamoto *et al.* 1999). Earlier reports had shown that induction of the CYP2B genes by PB is blocked by the protein phosphatase inhibitor okadaic acid (Sidhu and Omiecinski 1997). This inhibitor has since been shown to block nuclear accumulation of CAR by PB, suggesting that phosphorylation plays a role in this phenomenon (Kawamoto *et al.* 1999). This result is further supported by the finding that protein phosphatase 2A (PP2A) is recruited to the cytoplasmic CAR complex in the presence of inducers. The translocation event also appears to be mediated by a leucine-rich xenochemical response signal (XRS) within the C-terminal portion of the protein (Zelko *et al.* 2001). The prominent nuclear compartmentalization of CAR that is seen when overexpressed in cell lines is potentially due to a saturation of these cytoplasmic retention mechanisms.

After translocation to the nucleus the CAR/RXR heterodimer binds specific response elements upstream of target genes. Although the CAR/RXR heterodimer displays greatest sensitivity for DR-4 elements such as those found in the PBREM, it can also interact with a variety of other DNA target elements (Tzameli *et al.* 2000). The battery of CAR target genes include members of all three phases of xeno/endobiotic metabolism and clearance, such as certain CYPs, UDP-glucuronosyltransferase, sulfotransferase, glutathione-*S*-transferase, aldehyde dehydrogenase, and ATP-binding cassette (ABC) transporter families (Maglich *et al.* 2002; Ueda *et al.*

2002). Thus far, CAR response elements have been mapped in a number of corresponding human genes, including CYP2B6 (Sueyoshi *et al.* 1999; Wang *et al.* 2003), CYP3A4 (Goodwin *et al.* 2002; Sueyoshi *et al.* 1999), CYP3A5 (Burk *et al.* 2004), CYP2C8 (Ferguson *et al.* 2005), CYP2C9 (Chen *et al.* 2005; Ferguson *et al.* 2002; Gerbal-Chaloin *et al.* 2002), CYP2C19 (Chen *et al.* 2003), UGT1A1 (Sugatani *et al.* 2001), MDR1 (Burk *et al.* 2005), ALAS1 (Podvinec *et al.* 2004), Sult2A1 (Chen *et al.* 2007), and cathepsin E (Page *et al.* 2007b).

When the milieu of transcription factors bind their respective DNA response elements of a promoter, activation domains of these transcription factors interact with other proteins involved in assembling the transcriptional complex, known as the enhanceosome (Collingwood *et al.* 1999; Wolffe and Guschin 2000). The appropriate interaction of transcriptional activators is thought to result in the localized remodeling of chromatin, driven largely by posttranslational modifications of histone proteins within the core nucleosomal structures, thereby enhancing the further recruitment of accessory factors that in turn couple with the RNA polymerase II to drive transcription (Dilworth *et al.* 2000). CAR has been shown to interact with several transcription factors involved in chromatin remodeling. The list of coregulators includes the coactivators steroid receptor coactivator-1 (SRC-1) (Forman *et al.* 1998), PPARBP (Jia *et al.* 2005), transcriptional intermediary factor 2 (TIF2) (Lempiainen *et al.* 2005), acetyl-coenzyme A synthetase 2 (ACS-2) (Choi *et al.* 2005), and PPARY coactivator-1 α (PGC-1) (Shiraki *et al.* 2003) and the corepressors nuclear hormone receptor corepressor (NCoR) (Lempiainen *et al.* 2005) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) (Bae *et al.* 2004).

The coregulators associated with CAR at any point in time depend highly on the chemical environment. Interestingly, many of the chemicals that induce nuclear accumulation of CAR, including PB, do not bind the receptor. However, there are a number of chemicals that have been shown to regulate CAR through a direct interaction with its ligand binding pocket. The first CAR ligands to be discovered were the androgen metabolites androstanol and androstenol. These ligands act as repressors of the constitutive activity of CAR by inducing the release of coactivators; this unique mechanism has led to these ligands being termed inverse agonists. The concentration of androstanol and androstenol required to have this effect was in the low micromolar range and therefore unlikely to have any *in vivo* significance (Forman *et al.* 1998). Other ligands of CAR, such as 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) (Tzameli *et al.* 2000) and (6-(4-chlorophenyl)imidazo[2-1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO) (Maglich *et al.* 2003), function as activators by inducing nuclear translocation as well as recruiting coactivators or stabilizing the CAR/coactivator complex. TCPOBOP is specific to mouse CAR and also functions to increase the transcriptional activity of CAR above constitutive levels, an effect that has not been seen in human CAR. CITCO, on the other hand, is a specific human CAR agonist. The differences between these two chemicals highlight the divergence of the two orthologues. Other studies have shown that the antifungal agent clotrimazole (Moore *et al.* 2000) and the antinausea agent meclizine (Huang *et al.* 2004b) function as inverse agonists of human CAR (however, meclizine was shown to be a potent agonist of mouse CAR). Other activators and/or ligands of CAR include 5β-pregnane-3, 20-dione (Moore *et al.* 2000), artemisinin (Burk *et al.* 2005; Huang *et al.* 2004a), cyproconazole (Peffer *et al.* 2007), nonylphenol (Hernandez *et al.* 2007), phenytoin (Wang *et al.* 2004), carbamazepine, efavirenz, and nevirapine (Faucette *et al.* 2007). **Figure 2** provides a schematic representation of gene regulation by CAR.

Interestingly, the CAR gene itself appears to undergo transcriptional regulation. For example, interleukin-6 (IL-6) has been reported to rapidly and markedly decrease the expression of both CAR and PXR mRNAs in primary human hepatocyte cultures (Pascussi *et al.* 2000). Similarly, CAR is also upregulated in primary human hepatocytes in response to aryl hydrocarbon receptor (AhR) activation by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and benzo[*a*]pyrene (Patel *et al.* 2007). Therefore, control of CAR levels in the cell, via transcription and perhaps by posttranscriptional pathways, may be an important means of regulating CAR-mediated functional activity in the liver.

As is the case with most areas of biological study, use of viable experimental models is of strategic importance. In this respect, the PB induction response has traditionally been difficult to model using *in vitro* systems. Most hepatoma-derived cell lines do not exhibit the induction response and even many primary hepatocyte culture models only poorly reproduce the PB induction response as observed *in vivo*, and, as such, maintenance of the PB induction response appears to be a highly definitive marker of hepatocyte differentiation character (Sidhu *et al.* 2004). Certain well-defined primary

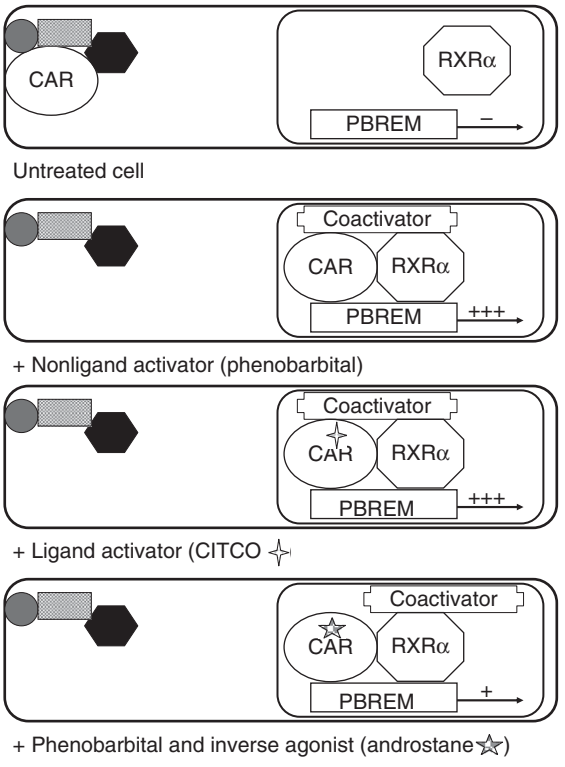

Figure 2 Diagram of gene regulation by CAR. In untreated cells, CAR is sequestered in the cytoplasm by a protein complex (top). After treatment with a chemical that activates CAR, it is released from this complex and translocates to the nucleus where it heterodimerizes with RXRα, binds to its response element, and recruits coactivators that in turn begin transcription of target genes. In the case of phenobarbital activation, this occurs without physical binding to CAR (second from top), which is in contrast to a CAR ligand such as CITCO, which physically binds the CAR ligand binding pocket (second from bottom). Inverse agonists such as certain androstane metabolites can repress target gene activation by binding to CAR and causing the displacement of coactivators, decreasing the transactivation potential of the complex (bottom).

culture models, both from rodent (Sidhu *et al.* 2004) and from human (Olsavsky *et al.* 2007; Page *et al.* 2007a), have been developed that appear to maintain robust PB response character as well as other differentiated features of hepatocytes *in vivo*.

2.10.3.3 Structure and Function

The original CAR clone encoded 348 amino acids resulting in a protein with three functional domains (**Figure 3**). The highly conserved DNA binding domain (C domain) resides on the NH_2-terminus followed by a hinge domain (D domain). The ligand binding domain (E domain) is highly divergent and makes up the COOH-terminal portion of the protein. This domain is responsible for ligand binding and dimerization with RXR, and contains the activation function 2 (AF2) helix required for coactivator recruitment on the extreme far end of the COOH-terminus of proteins. CAR lacks the NH_2-terminal A/B domain that would normally include the activation function 1 (AF1) motif as well as the hypervariable F domain that resides on the COOH-terminus of most nuclear hormone receptors (Baes *et al.* 1994).

In 2004, the crystal structures were resolved for the ligand binding domain of both human (Xu *et al.* 2004) and mouse (Shan *et al.* 2004; Suino *et al.* 2004) CAR. These studies have yielded a great deal of insight into the structural features that maintain CAR in the active conformation. The secondary structure consists of eleven α helices, two 310 helices located between helix-1 and helix-3, and three β strands. In human CAR, four residues (six residues in mouse CAR) (Suino *et al.* 2004) between helix-10 and the AF2 helix form a single turn, referred to as helix X. It is hypothesized that helix X, which is separated from the AF2 helix by a single methionine residue, maintains CAR in the active conformation by providing a rigid structure that limits the conformational freedom of the AF2 helix. Furthermore, helix X and the AF2 sit atop 4 amino acids that shield

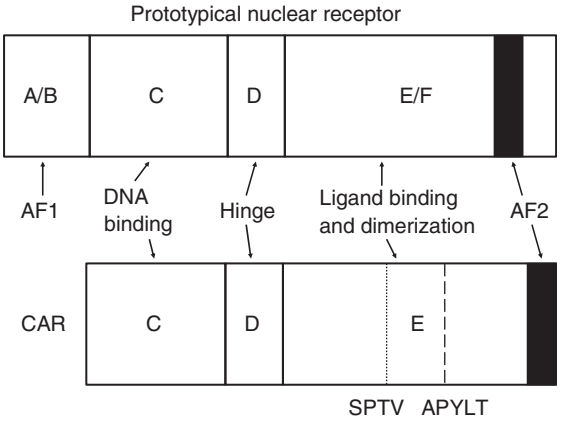

Figure 3 Diagram of the CAR domain structure compared to a prototypical nuclear receptor. CAR lacks an N-terminal A/B domain and the corresponding AF1 motif. The DNA binding domain (C domain) of CAR makes up the N-terminal portion of the protein followed by the hinge domain (D domain) and by the C-terminal ligand binding domain (E domain), which also functions as the dimerization surface. Unlike most nuclear receptors, CAR lacks a C-terminal extension after the AF2 helix (sometimes referred to as the F domain). The diagram also shows the approximate location in human CAR of the 4-amino-acid (SPTV) insertion of CAR2 (dotted line) and the 5-amino-acid (APYLT) insertion of CAR3 (dashed line).

the CAR ligand binding pocket, limiting the effects that ligands can have on the receptor (Xu *et al.* 2004). The crystal structure of the ligand binding domain of murine CAR reported by Shan *et al.* (2004) showed that the receptor bound to androstenol providing insight into the mechanism of inverse agonism. Unlike typical nuclear receptor ligands, androstenol does not contact the AF2 helix. Instead, it appears that androstenol disrupts CAR's active conformation by causing a kink in between helix-10 and helix-11 and disrupting the features that hold the AF2 helix in place (Shan *et al.* 2004). It is not yet known if inverse agonists affect human CAR in the same way although it would seem that this is likely to be the case.

2.10.3.4 Physiological Roles

Although CAR was originally characterized as a xenobiotic sensor regulating hepatic drug metabolizing genes in response to exogenous chemicals, CAR activity has been implicated additionally in the metabolism of endogenous compounds including steroids, heme (Xie *et al.* 2003), bile acids (Guo *et al.* 2003), and thyroid hormone (Maglich *et al.* 2004; Qatanani *et al.* 2005). Studies utilizing the CAR knockout mouse have demonstrated that CAR is also involved in the processes that result in acetaminophen- (Zhang *et al.* 2002) and bile acid- (Zhang *et al.* 2004) induced liver toxicity. There is further evidence that CAR activity impinges upon signaling pathways that control food consumption (Qatanani *et al.* 2004) and metabolic changes during periods of fasting (Ding *et al.* 2006). In large part, the effects that CAR exerts on these processes are dependent on the receptor's ability to modulate hepatic gene expression.

Some of these studies along with the observation that chronic PB treatment leads to a decrease in blood glucose levels in diabetic patients (Konno *et al.* 2008; Lahtela *et al.* 1985) have led to more recent reports identifying CAR as a regulator of hepatic energy metabolism. In the fasted state the liver can increase plasma glucose levels through gluconeogenesis and glycogenolysis. It appears that activation of CAR can repress this process through direct interactions with FoxO1 (Kodama *et al.* 2004) and PGC-1α (Miao *et al.* 2006), transcription factors that respond to insulin and glucagon to regulate genes for the rate-limiting enzymes of the gluconeogenesis and glycogenolysis pathways, glucose-6-phosphatase and phosphoenol-pyruvate carboxykinase 1. We are only beginning to understand this function of CAR and further work will be needed to fully elucidate this important biological role that may prove to be of great therapeutic value in the battles against diabetes and obesity. There are two recent reviews that have been written on this topic that we refer the reader to for further information (Konno *et al.* 2008; Moreau *et al.* 2008).

2.10.3.5 Alternative Splicing of the Human CAR Gene

It is generally viewed that in the first step of transcription a pre-mRNA molecule is transcribed by

RNA polymerase II as an exact RNA copy of the genomic DNA, including all introns and exons. The pre-RNA molecule is then spliced by the spliceosome complex generally resulting in the removal of the introns and ligation of the exons to form the mature mRNA. During this processing, the RNA can be put together in a multitude of ways through the use of alternate splice donor and acceptor sites (Black 2003). Recent studies have demonstrated that RNA splicing can occur cotranscriptionally, with the splice sites identified by the spliceosome while downstream exons still await their synthesis (Hertel 2008; Kornblihtt 2007). These mechanisms of alternative splicing make it possible to produce a wide array of transcripts from a single pre-mRNA molecule. An estimated 70% of all human genes undergo alternative splicing (Hertel 2008; Johnson *et al.* 2003; Modrek and Lee 2002) and therefore this process appears to represent a mechanism through which higher organisms increase genetic complexity. For further reading on alternative RNA splicing, the reader is directed to a recent series of mini-reviews from *The Journal of Biological Chemistry* (Ben Dov *et al.* 2008; Fedor 2008; Hertel 2008; House and Lynch 2008; Stamm 2008).

Recently, our laboratory and others have identified more than 20 alternatively spliced CAR transcripts (Auerbach *et al.* 2003, 2005, 2007; Jinno *et al.* 2004; Lamba *et al.* 2004). Two of these variants, termed CAR2 and CAR3, contain small insertions, 4- and 5-amino-acid insertions, respectively, that lie within the ligand binding domain of the receptor (see **Figure 3**) and are predicted to posses unique biology (Arnold *et al.* 2004; Auerbach *et al.* 2003; Jinno *et al.* 2004). The variant receptors may have altered affinities for RXRα and distinct DNA interaction profiles (Arnold *et al.* 2004; Auerbach *et al.* 2003). CAR2 and CAR3 transcripts were reported to comprise 6–10 and 40%, respectively, of the total CAR transcript in human liver (Jinno *et al.* 2004); however, more recent data from our laboratory indicate that CAR2 and CAR3 comprise approximately 30 and 20%, respectively, of the CAR receptor pool in the liver (DeKeyser and Omiecinski, unpublished results).

The CAR2 transcript results from the use of an alternative splice acceptor site in intron 6, leading to the insertion of 12 additional nucleotides (Auerbach *et al.* 2003). This transcript encodes a protein containing an additional 4 amino acids (SPTV) that are predicted to extend helix 6 of the ligand binding domain and potentially affect the structure of the ligand binding pocket (Auerbach *et al.* 2007). Although CAR2 was reported initially to retain a more modest ability to transactivate CAR-responsive reporters (Arnold *et al.* 2004; Auerbach *et al.* 2003), more recent results indicate that this variant possesses transactivation potential equal to CAR1 under appropriate conditions, including the ability to be activated by CAR2-selective ligands, such as di(2-ethylhexyl) phthalate (Auerbach *et al.* 2007) (DeKeyser *et al.* 2009). Earlier studies of CAR2 demonstrated that clotrimazole deactivated the receptor, whereas CITCO produced a weak, albeit significant, activation of CAR2 (Jinno *et al.* 2004) – a result that is contrary to mammalian two-hybrid studies published separately (Auerbach *et al.* 2003).

CAR3 is produced through the use of an alternative splice acceptor site in intron 7 resulting in the insertion of an additional 15 nucleotides (Auerbach *et al.* 2003). The CAR3 transcript encodes a protein containing 5 additional amino acids (APYLT) in the highly conserved loop 8–9 of the ligand binding domain (Auerbach *et al.* 2003). CAR3 exhibits ligand-dependent interaction with coactivators recruited by the human CAR1 agonist CITCO (Arnold *et al.* 2004). CAR3 has also been shown to transactivate an optimized DR4 response element as well as reporters containing promoters from the endogenous CYP2B6 and CYP3A4 genes (Auerbach *et al.* 2005). Transactivation was ligand dependent and enhanced by the overexpression of RXRα (Auerbach *et al.* 2005). As a ligand-dependent form of CAR, CAR3 shows potential as a diagnostic tool for *in vitro* screening of possible CAR ligands and has already been used for this purpose in one study (Faucette *et al.* 2007).

2.10.4 Cellular Differentiation and Signaling

Despite major advances in identifying key receptor pathways and transcriptional events involved in the PB induction process, many additional details of this regulatory response remain to be elucidated. These include details of the signaling processes necessary for determining the differentiation status of the cell.

As alluded to earlier, the PB induction process is tightly coupled to a highly differentiated hepatic phenotype. Although some evidence for a PB induction response has been obtained in intestinal enterocytes and in certain brain regions, the magnitude of the expression response in the latter cell types is substantially less than that occurring in the liver (Schilter *et al.* 2000). Historically, it has been difficult

to model the PB responses that occur in the intact liver. Hepatoma cell lines are largely refractive to PB induction; moreover, as these cells become 'immortalized,' they rapidly and permanently lose many differentiation features, often including the capacity to express other genes that are responsible for xenobiotic metabolism (Schuetz *et al.* 1988; Sidhu and Omiecinski 1995; Waxman and Azaroff 1992).

Research from our laboratory has established the importance of a variety of defined conditions that enable cultured hepatocytes to exhibit most features inherent in the differentiated adult liver phenotype, including *in vivo*-like responsiveness to PB inducers (Sidhu *et al.* 1993, 1994). Appropriately maintained cultures of primary hepatocytes retain differentiated features and are therefore valuable models for assessing liver-specific responses to pharmacological and toxicological agents. Important parameters required for the maintenance of these responses include the provision of extracellular matrix contacts for cultured hepatocytes, use of a serum-free medium formulation, and inclusion of physiological concentrations of insulin and dexamethasone (Olsavsky *et al.* 2007; Page *et al.* 2007a; Sidhu *et al.* 2001). Hepatocytes are quite sensitive to their culture environment such that suboptimal conditions activate prototypical stress pathways, including stimulation of stress activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) and MAPK phosphorylation, with the resultant nuclear recruitment of the stress-associated transcription factors AP-1 and NF-κB (Sidhu *et al.* 2001). For example, elevated concentrations of several commonly used PI3 kinase inhibitors enhance hepatocyte cytotoxicity and result in the elevated expression of CYP2E1 mRNA (Sidhu *et al.* 2001). Stress-related responses arising from chemical or oxidant exposures, or suboptimal levels of extracellular matrix, insulin, and/or dexamethasone, are associated with a marked downregulation of several hepatocyte-enriched nuclear transcription factors, factors that are critical for the maintenance of hepatic phenotype, including maintenance of PB inducibility. Unfortunately, many investigators have historically deployed culture models that compromise the differentiated character of hepatocytes, therefore limiting the true potential of this model system.

2.10.5 Summary

The CAR plays a major role in the body's ability to mount a defense against potentially toxic endogenous chemicals. Unfortunately, this defense can result in a number of unwanted side effects, generally in the form of adverse drug reactions. Studies have indicated that more than 2.2 million hospitalized Americans suffer adverse drug reactions each year and that approximately 100 000 die unintentionally from administration of medications (Lazarou *et al.* 1998). This trend seems likely to continue due to the increased use of multiple drug regimens in patients (Gorard 2006). A complete knowledge of the systems involved in drug and xenobiotic metabolism is paramount if we hope to predict and prevent these outcomes.

Investigations into the PB induction response have added a great deal to our understanding of drug metabolism. The discovery and characterization of CAR as a mediator of this response has provided novel insight into potential mechanisms of adverse drug reactions. Furthermore, it has opened the door for further studies on liver biology, most notably nuclear receptor-mediated regulation of hepatic energy metabolism, an important factor in diabetes and obesity. As described in Section 2.10.3.2, there are marked differences between human and rodent CAR especially in terms of the ligands that they bind. These differences have important implications regarding the model systems that need to be employed for studies that aim to elucidate the function of CAR in humans. This issue is further complicated by the existence of species differences and alternatively spliced variants of human CAR, many of which we currently know very little about. With these caveats in mind it is likely that future studies into CAR will not only further aid our ability to assess drug and chemical safety but may also yield novel therapies for human disease.

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