8 Transcriptional Regulation of Cytochrome P450 Genes

EMILY A. FRASER, COLIN J. HENDERSON, and C. ROLAND WOLF
Division of Cancer Research, University of Dundee, Medical Research Institute, Ninewells Hospital & Medical School, Dundee, UK

8.1 INTRODUCTION

Our mechanistic understanding of the transcriptional regulation of cytochrome P450 (P450) expression has increased significantly in recent years, with the discovery and characterization of "orphan" nuclear receptors such as the constitutive androstane receptor (CAR) and the pregnane X receptor (PXR). The role of these receptors as mediators of the inductive effects of xenobiotics continues to be the subject of intensive investigation and has added a further layer of complexity to an already burgeoning area of research.

It has become clear that significant variability exists at almost every level of the processes that contribute to the ultimate functionality of P450 proteins, and transcriptional regulation is no exception. Expression of these proteins is controlled by transcription factors that not only vary in their expression level and the degree to which they bind and respond to different classes of ligands but are also themselves subject to alternative splicing, producing variant receptor forms with different spatiotemporal expression and functionality. It is clear that only by gaining a detailed understanding of these receptors will we have a greater appreciation of the intricacies of transcriptional regulation of P450s.

This chapter concentrates on those transcription factors involved in xenobiotic-induced expression, giving an overview of regulatory interactions and raising issues that have a bearing on transcriptional regulation, such as polymorphisms, splice variants, and species specificity. However, it is important to note that other transcription

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factors that are not covered in this review - including the peroxisome proliferator activated receptors α and γ, the vitamin D receptor, and the oestrogen receptors α and β - are also involved in regulation of endogenous and basal cytochrom P450 expression.

8.2 TRANSCRIPTIONAL REGULATION OF CYTOCHROME P450s

8.2.1 Pregnane X Receptor (PXR)

The pregnane X receptor (PXR, also known as SXR, PAR, and NR1I2) is the most recently identified of the three main regulators of xenobiotic-induced CYP expression, with the mouse ortholog having been first identified by Kliewer *et al*. in 1998, closely followed by the identification of the human form by Blumberg *et al*. [1,2]. A ligand-activated orphan nuclear receptor was named the pregnane X receptor because of its activation by pregnenolone derivatives. Acting as a xenosensor, PXR is one of the key controllers of drug metabolism, in particular, for the expression of the CYP3A enzyme isoforms. In addition to the CYP3A enzymes, PXR, bound as a heterodimer to its partner, the retinoid X receptor (RXR), regulates a wide variety of P450s, as well as phase II enzymes and phase III drug transporters (Table 8.1). PXR is also promiscuous, being able to accommodate and be activated by a wide range of different ligands, both endogenous and exogenous (Table 8.2). This protein is expressed in many tissues, including those of heart, colon, stomach, and certain brain regions, although it is primarily expressed in the liver and small intestine [3]. Several splice variants have also been identified, which affect PXR-mediated transcriptional regulation (Section 8.3.3).

Flexibility in the ligand-binding domain (LBD) of PXR is the reason for its wide substrate specificity, with the binding pocket able to expand from its resting 1150 $Å^3$ to more than 1600 $Å^3$ when ligand bound. Large molecules, such as the macrolide antibiotic rifampicin (RIF), can therefore activate the receptor using an induced-fit

TABLE 8.1 Examples of Human Cytochrome P450s Regulated by Human PXR, CAR, and AHR

Gene	PXR	CAR	AHR	References
CYP1A1	—	—	↑[a]	[4][a]
CYP1A2	↑	↑[b]	↑[a]	[5][b] and [4][a]
CYP1B1	↑	↔	↑	[6,7]
CYP2A6	↑[b]	↑	—	[8][b]
CYP2B6	↑	↑	↔	—
CYP2C8	↑	↑	—	[9]
CYP2C9	↑	↔	↔[b]	[10] and [4][b]
CYP2C19	↑	↑	—	—
CYP3A4	↑	↑	↔	—
CYP7A1	↓[b]	↓[a]	↔[c]	[11],[b] [12],[a] and [4][c]
CYP8B1	↓[b]	↓[p]	—	[11][b] and [13]
CYP24A1	↑	↑	—	[14]

Bold arrows indicate strong induction.

↑, induced; ↓, repressed; ↔, no change/basal expression; [p], putative interaction in humans. a, b, or c on each line refers change in nuclear receptor expression to the corresponding citation.

Source: Unless otherwise stated, data was extracted from Ref. 15.

TABLE 8.2 Example Modulators of Human PXR, CAR, and AHR

Class	Compound	PXR	CAR	AHR	References
Classical	Rifampicin	++			PXR/CAR: [7,15–21] AHR: [6,22]
	CITCO		++		
	TCDD			++	
	3-Methylcholanthrene			++	
Drug	Omeprazole	+		+	
	Thiabendazole			+	
	Nicotine			+	
	Caffeine			+	
	Paclitaxel	+			
	Methadone	+	+		
	Clotrimazole		−		
	Ketoconazole	−	−		
	17α-Ethinylestradiol	+			
	Mifepristone	+			
	SR12 813	+			
	Phenytoin	+			
	Primaquine	+			
	Spironolactone	+			
Herb	Hyperforin	++			
	Cryptotanshinone	+			
	Artemisinin	+	+		
	Ginkgo biloba	+	+		
	Schisandrin A-C	+			
Dietary	β-Carotene	+		+	
	Vitamin E	+			
	Flavonoids			±	
	Curcumin			+	
Endogenous	Androstanol		+		
	Corticosterone	+			
	17β-Estradiol	+	+		
	Vitamin K_2	+			
	Tryptophan metabolites			+	
	Bilirubin			+	
Environmental/ industrial	Benzyl butyl phthalate	+			
	Chlordane	+			
	Phthalic acid (DHEP)	+	+		
	Nonylphenol	+	+		
	Polychlorinated biphenyls	+			
	Toxaphene	+			
	Triclopyr	+			
	Benzo(*a*)pyrene			+	
	1-Methyl-1-phenylhydrazine			+	

Blank spaces indicate no data is available.

++, very strong agonist; +, agonist; –, antagonist (or inverse agonist, CAR only); ±, antagonist/agonist, dependent on cell context.

For further information see reviews [6,15–17].

mechanism, which allows molecules to adopt various binding orientations before selecting the optimal configuration [23–25]. The LBD is predominantly hydrophobic in character, with Met243, Ser247, Gln285, Trp299, His407, and Phe420 commonly interacting with ligands [26,27]. Site-directed mutagenesis studies have indicated that even small changes in the LBD can have significant effects on the ligand-induced activation of PXR [25,26]. Interspecies variability in this region therefore has significant effects on the ligand activation profile (Section 8.3.2).

Nuclear translocation is a controlling factor in PXR-mediated P450 induction. However, because of difficulties with investigating translocation *in vitro*, namely, when PXR is overexpressed it spontaneously translocates to the nucleus [28], data regarding this mechanism remains limited. It has been reported that when inactive, PXR is bound in a complex with cytoplasmic CAR retention protein (CCRP) and HSP90, which retains the receptor in the cytoplasm [29,30]. Although the mechanism underlying PXR dissociation from the complex and translocation to the nucleus has not yet been elucidated, it is likely that a mechanism similar to that governing CAR dissociation from the retention complex is involved (Section 8.2.2). Once released from the cytoplasmic retention complex, PXR nuclear localization signals (NLSs) are targeted by importin-α proteins for nuclear import [29].

Upon entering the nucleus, PXR heterodimerizes with its binding partner, the RXRα, before binding to response elements in the promoter and enhancer regions of target genes (Fig. 8.1). PXR heterodimers preferentially bind direct repeat sequences possessing the half site AG(G/T)TCA separated by three nucleotides (DR3) and everted repeats of the half site separated by six nucleotides (ER6), that is, AG(G/T)TCAnnnAG(G/T)TCA and TGA(A/C)CTnnnnnnAG(G/T)TCA, respectively [15,16]. However, they are also able to bind other recognition sites, such as DR4, DR5, and ER8 motifs, although with lower affinity [31], thus accounting for the cross talk between receptors, which is an important contributor to P450 regulation.

A key component of transcriptional activation by nuclear receptors is the recruitment of coactivators and corepressors to promoter regions, following DNA binding. Coactivators of PXR, CAR, and AHR (aryl hydrocarbon receptor) are given in Table 8.3. As an example, PXR is a binding partner for the p160/SRC (steroid receptor coactivator) coactivator family, which recruits histone acetyltransferase complexes, such as cAMP response element binding protein/p300 (CREB), thus providing access to DNA strands for RNA-polymerase-catalyzed transcription [25]. These coactivators possess three LXXLL motifs that bind to the AF-2 helix, interacting with two charged residues on the receptor surface (Lys259 and Glu427) to form a charge clamp [24]. Crystal structures of SR12813 in complex with PXR and SRC-1 indicate that in the presence of coactivator, the ligand will bind in a single orientation. The combination of ligand and SRC-1 binding stabilizes the protein structure to restrict the flexibility of the LBD once an active ligand conformation has been achieved [25]. In contrast, corepressors are thought to bind in the absence of ligand, or in the presence of antagonists, retaining the AF-2 helix in a nonactive conformation [16]. Corepressors of PXR include the nuclear receptor corepressor (NCoR) and the silencing mediator of retinoid and thyroid hormone receptor (SMRT). SMRT possesses both ID1 and ID2 interacting domains, which consist of a CoRNR box (I/LXXI/VI) and the motif LXXXIXXXI/L, respectively [32]. PXR binds to the ID2 motif preferentially over the ID1, with the key interacting residues being Lys259, Gly270, and Pro423 of the PXR LBD and Arg2347, Lys2348, and Leu2350 of SMRT [32]. Site-directed mutagenesis studies show that mutation

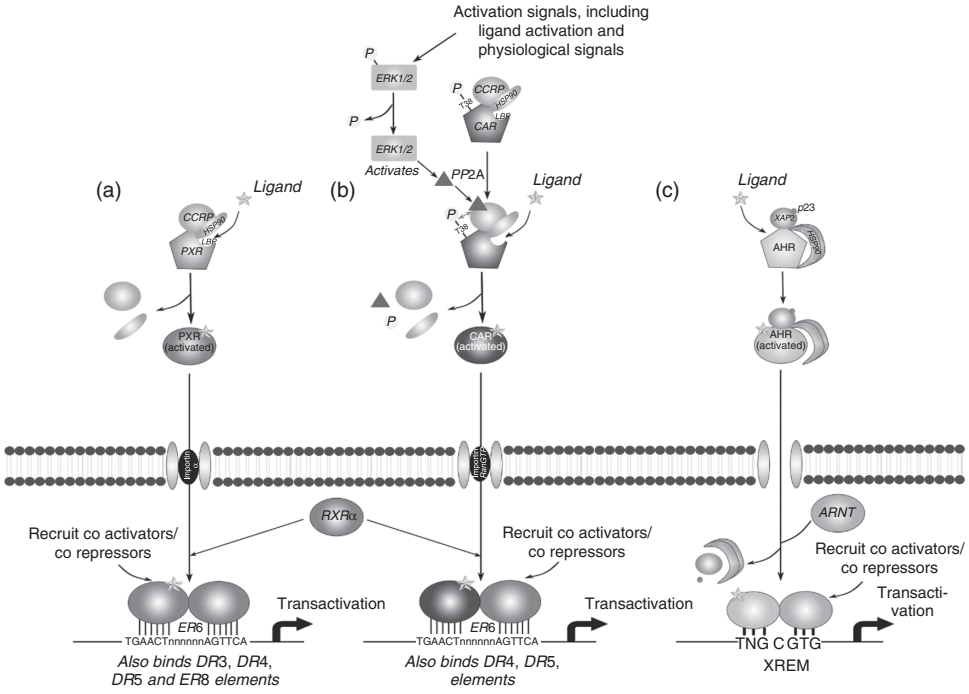

Figure 8.1 Activation mechanisms of (a) PXR, (b) CAR, and (c) AHR. (See color insert.)

TABLE 8.3 Coactivators and Corepressors Associated with PXR, CAR, and AHR

Coregulator Type	Coregulator	PXR	CAR	AHR	References
Coactivators	SRC-1	*	*	*	AHR: 33,34 PXR/CAR: 12, 15, 16, 35
	NCOA2 (GRIP1, SRC-2)	*	*	*	
	p/CIP (SRC-3)	*		*	
	P300			*	
	CBP			*	
	RIP 140	*		*	
	CARM1			*	
	PRMT1			*	
	PGC-1α	*	*		
	PBP	*	*		
	ASC-2		*		
	SMC-1		*		
Corepressors	ANKRA2			*	
	SMRT	*		*	
	NCoR	*	*		
	SHP	*			

[a]Corepression of AHR via the AHR repressor [36].

of charged residues within the PXR LBD (Arg410Asn, Asp205Ala, Glu321Ala, and Arg413Ala) increases the binding affinity for corepressors, indicating a core role for these residues in the maintenance of basal PXR activation [32]. Acting in concert, the recruitment of coregulators therefore controls the expression of target genes, such as P450s, by regulating access of RNA polymerases to the promoter regions.

A further aspect of PXR-mediated regulation of P450s is posttranslational modification of PXR by phosphorylation. This modification is known be critical to the correct function of other nuclear receptors, including CAR and AHR, but, until recently, had not been subjected to a systematic analysis with respect to PXR. Research from the Staudinger group has indicated that protein kinase A (PKA) interacts with PXR, but in a species-specific manner, synergizing with ligand activation in mice but repressing ligand-induced activation in rats and humans [37]. Protein kinase C (PKC) and CDK2 signaling has also been implicated. A recent mutagenesis study, in which 18 serine and threonine residues identified by either *in silico* site prediction or comparison with other receptors, has shed more light on the role of phosphorylation in PXR activity. These studies have shown that the phosphorylation state of certain key residues (Ser8, Thr57, Ser208, Ser305, Ser350, and Thr408) is involved in the control of heterodimerization, DNA binding, corepressor recruitment, and nuclear translocation [38]. The phosphorylation state of PXR therefore appears to be important in controlling gene expression. However, these observations require further studies to fully understand the role of phosphorylation in PXR function.

In summary, PXR is a key controller of P450 regulation, being associated with a wide variety of target genes. It also has an important role in many endogenous processes, including control of glucose homeostasis and the cholesterol and bile acid metabolism [15,16]. However, most importantly from a pharmaceutical perspective

is its role as the key regulator of the CYP3A enzymes, which are responsible for the metabolism of a significant proportion of currently used pharmaceuticals, and as a consequence involved in a significant number of drug–drug interactions [39]. As a key regulator of P450s, xenobiotics that interact with PXR could have a profound effect on their own metabolism as well as on the metabolism of other drugs given concomitantly. The promiscuity of PXR in terms of its ligand interactions and the wide range of genes it transactivates can result in severe drug–drug interactions [40–43]. As a consequence, PXR transactivation is investigated as a part of preclinical drug development programs. Regulation of this signaling pathway is still incompletely understood, although it is clear that it involves a complex interaction between coregulators, protein structure, and phosphorylation state.

8.2.2 Constitutive Androstane Receptor (CAR)

Initially identified in humans in 1994 as MB67 [44], and in the mouse in 1997 [45], the constitutive androstane receptor (CAR, NR1I3) is well established as a key regulator of P450 expression [16,39,46–57]. The genes targeted by CAR are numerous and varied, encompassing P450s (Table 8.1), phase II enzymes such as UDP-glucuronyltransferases, and phase III drug transporters, with CYP2B6 being prototypically induced (Cyp2b10 in mice, CYP2B1/2 in rat) [58,59]. Lacking a physiological ligand, this protein was also classed as an orphan nuclear receptor. However, as a nuclear receptor, CAR has some unusual properties, having both a different protein structure and displaying constitutive transcriptional activity [60].

As a consequence of the constitutive activity of CAR, molecules such as androstanol and androstenol have been referred to as *inverse agonists* because they actively repress constitutive CAR activity instead of antagonizing in the classical sense [61]. While not as promiscuous as PXR, CAR can be activated by numerous compounds (Table 8.2), but few operate by binding directly to CAR. Its constitutive basal activity means that it can transactivate genes without ligand binding, in response to externally acting signals that induce nuclear translocation [62]. This indirect activation pathway is a key mechanism in CAR-mediated transactivation, with phenobarbital (PB) induction of CYP2B being the classic example, and is suggested to be the major activation pathway for this molecule [63,64]. These phenomena are dependent on several key structural features of CAR and the increasingly understood mechanism of nuclear translocation, which are considered below.

Structurally, a protein consists of three domains: (i) a highly conserved N-terminal DNA-binding domain, (ii) a hinge region, and (iii) a variable C-terminal domain associated with ligand binding (AF-2 domain), dimerization with the CAR-binding partner (RXRα), and transcriptional activation [54]. The ligand-binding pocket (LBP) is significantly smaller than that of PXR at approximately 675 $Å^3$ in humans and is predominantly hydrophobic in nature. The conformation of the AF-2 domain within the LBP is thought to be vital to the constitutive activity of the protein, although it is not accessible for ligand binding, being protected by the side chains of Phe161 (also key in constitutive activity), Asn165, Phe234, and Tyr326, which are conserved across all mammalian orthologs of CAR [65,66]. It has also been suggested that disruption of the AF-2 conformation is responsible for switching CAR to its inactive form, such as is seen upon inverse agonist interaction [27].

Although CAR is described as constitutively active, it is unable to activate transcription of downstream genes in the absence of an external signal because of regulatory mechanisms controlling its translocation to the nucleus. In the absence of ligand or indirect activation, CAR is sequestered in the cytoplasm, bound to a complex consisting of CCRP and HSP90, along with other accessory proteins [67,68]. In response to direct or indirect ligand challenge, NLSs are activated, resulting in dissociation from this complex. Once the ligand is activated and released from the cytoplasmic retention complex, active CAR is translocated into the nucleus via an importin/Ran-GTP mediated process involving NLS binding to IPO13 [69].

The molecular trigger for, and underlying mechanism of, nuclear translocation is still unclear, although recent research has implied an essential role for cellular signaling pathways and phosphorylation status, especially in indirect control [28]. Although the role of phosphorylation state in CAR translocational control had been described previously, it was not until 2009 that the Thr38 amino acid was identified as the key residue in regulation of human constitutive androstane receptor (hCAR) translocation. Phosphorylation of Thr38 by PKC inactivates CAR by destabilizing the helix containing the C-terminal region of the first zinc finger, resulting in ablation of DNA binding [70]. However, on Thr38 dephosphorylation, the disrupted helix regains its stability, resulting in nuclear translocation and transactivation of downstream genes. It has been suggested that an essential process in PB-induced activation is the recruitment of protein phosphatase 2A (PP2A) to the retention protein complex in mouse hepatocytes, subsequently inducing translocation [71,72]. PP2A has therefore been suggested as a candidate for the dephosphorylation of this residue, acting in concert with other cofactors recruited to the CCRP/HSP90 complex. In addition, a recent report by Koike *et al*. identifies extracellular signal-regulated kinase (ERK) 1/2 as the endogenous signal controlling sequestration of CAR in the cytoplasm, with dephosphorylation of ERK 1/2 being sufficient to induce nuclear translocation [73]. AMP-activated protein kinase has also been suggested to influence CAR localization following PB treatment, although currently available data is conflicting [64,74–77]. Mutoh *et al*. suggest a model for both direct and indirect activators, in which PKC phosphorylates and PP2A dephosphorylates Thr38 to control nuclear translocation. The signal controlling the phosphorylation state of this residue appears to be extracellular signal-regulated kinase (ERK) 1/2 [70,73]. However, a membrane-bound regulatory subunit of protein phosphatase 1b, PPP1R16A, has recently been shown to interact with CAR, inducing translocation, which agrees with a report which localizes CAR at the cell surface in addition to the cytoplasm [78,79]. This suggests another layer of complexity in controlling nuclear translocation.

Once in the nucleus, CAR binds to RXRα in a head to tail arrangement and the resulting heterodimer binds to response elements in the promoter of target genes (Fig. 8.1). CAR will preferentially bind to three binding motifs: (i) DR4 (e.g., AGTTCAnnnnAGTTCA), (ii) DR5 (e.g., AGTTCAnnnnnAGTTCA), and (iii) ER8 (e.g., TGAACTnnnnnnnnAGTTCA). However, CAR can bind other motifs, such as ER5–ER10 types, but with lower affinity [15,80]. Unusually, CAR is also able to bind to DNA as a monomer, having a preference for DR4 motifs [80]. The optimal binding site for monomeric CAR was found to be AGAGTTCA. Binding to these motifs as a monomer is as strong as that of the T_3 thyroid hormone receptor, and the preference for monomeric binding is more marked in humans than in mice. However, if the 5′ nucleotide flanking sequences contain pyrimidine nucleotides, the binding

tendency of monomeric CAR is greatly reduced. All these binding motifs have been identified in the phenobarbital response element (PBREM) commonly found in CAR target genes [80]. Once bound, activated CAR will recruit coactivators/corepressors to modulate gene expression (Table 8.3).

Although this chapter has concentrated on the role(s) of CAR in xenobiotic metabolism, it is important to remember that CAR also has many endogenous functions. It is particularly important in control of lipid metabolism and energy homeostasis, appearing to act as an energy sensor, and influencing metabolism accordingly [17,81–86]. CAR is also involved in the control of bilirubin metabolism and heme biosynthesis, as well as bile acid and steroid/thyroid hormone homeostasis. For a recent review of this area, see di Masi *et al*. [15]. The range of endogenous roles with which CAR is involved means it is vital to consider the potential for functional cross talk between endogenous control and xenobiotic metabolism. For instance, the energy state of an organism can have significant effects on the uptake and pharmacokinetics of a drug because nutritional state can influence the induction of P450s by CAR [17]. Other considerations relating to CAR-induced xenobiotic metabolism include differential regulation due to species specificity, gender-specific induction, and the circadian control. Species specificity is discussed in more detail later in this chapter. Circadian control is a potentially important determinant of CAR expression and induction. CAR exhibits a circadian expression profile in mice because of its regulation by the PAR-domain basic leucine zipper transcription factors DBP (albumin D-box binding protein), TEF (thyrotroph embryonic factor), and HLF (hepatic leukemia factor), all of which are regulated by core components of the circadian machinery [87]. This results in a temporal induction of CAR target genes. Since downstream genes, such as P450s, are likely to display circadian expression profiles as a result, it is understandable that the clinical profile of certain drugs, such as cyclophosphamide and mitoxantrone, improves when dosing time in terms of circadian cycle is considered [87]. This feature should always be considered when investigating compounds that are likely CAR interactors, particularly in mouse models.

Overall, CAR regulation of P450 expression is complex, involving structural activation, regulation by signaling pathways, and interaction with various factors influenced downstream by CAR, including energy metabolism. It is, therefore, important when investigating compounds that appear to induce P450s via a CAR-dependent mechanism to consider that the endogenous control of CAR and the way in which it influences other homeostatic mechanisms, such as energy homeostasis, can have a significant effect on P450 expression profile and thus xenobiotic metabolism.

8.2.3 Aryl Hydrocarbon Receptor

The AHR has been known significantly longer than PXR and CAR, having been first identified in 1976 as the cytosolic receptor mediating 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-dependent induction of aryl hydrocarbon hydroxylase activity [88]. However, unlike PXR and CAR, the AHR is not a nuclear receptor but is the only known ligand-activated member of the basic helix-loop-helix (bHLH)/*p*eriod (PER)-*a*ryl hydrocarbon receptor nuclear translocator (ARNT)-*s*ingle minded (Sim) (bHLH-PAS) family of transcription factors. AHR ligands can be classified as synthetic, for example, dioxins and halogenated polycyclic aromatic hydrocarbons, and naturally occurring, such as flavonoids, indoles, and arachidonic acid metabolites [6,22,89]. As

a family, these proteins are implicated in the regulation of physiological processes, including organ development, metabolism, and stress and immune response [90]. However, one of the key roles of AHR is the control of xenobiotic metabolism.

AHR is responsible for the transactivational control of a variety of P450s (Table 8.1), most notably CYP1A1, CYP1A2, and CYP1B1, with CYP1A1 being considered a model enzyme for studying AHR-mediated gene activation. In order to modulate transcription, AHR heterodimerizes with its binding partner ARNT [33]. The transcriptional activation domains (TADs) of both molecules, once activated by ligands, activate transcription by inducing DNA binding via a subdomain N-terminal to the HLH domain. Both AHR and ARNT possess one TAD in the C-terminal region, with both containing glutamine and hydrophobic residues. However, the TAD of AHR also has acidic characteristics, whereas that of ARNT is rich in serine, threonine, and proline [91]. It is worth noting that while the N-terminal domain of AHR is well conserved across species, the C-terminal domain, and thus the TAD, is significantly divergent (58% identity between human and mouse forms) [92–94]. As a consequence, there are significant species differences in response to ligands (see Section 8.3.2 for details). Following activation, the AHR/ARNT heterodimer binds to the xenobiotic response element (XRE) in the promoter and/or enhancer regions of target genes (Fig. 8.1). Unlike classical bHLH proteins, the AHR/ARNT heterodimer binds to an asymmetric recognition site, binding to the core sequence of the XRE—*TNG*C*GTG* [92,95,96]. It has also been suggested that nucleotides adjacent to, but not part of, the core binding sequence are important for regulation of some genes [95]. Following DNA binding, AHR/ARNT recruits coactivators (Table 8.3) and other proteins, such as Mediator D and TFIID, resulting in chromatin remodeling and recruitment of RNA polymerases and in the initiation of gene expression (for a review of DNA binding, see Ref. 95).

Nuclear translocation is also a key step in the control of AHR-induced transcriptional regulation. AHR protein, which is inherently unstable in the absence of ligand, is stabilized in the cytoplasm by a complex consisting of two molecules of HSP90 and one each of hepatitis B virus X-associated protein 2 (XAP2) and the co-chaperone p23 [97–99]. The E3 ubiquitin ligases, C-terminal of HSP70 interacting protein (CHIP), and Cullin 4B also bind to AHR, which could explain the rapid degradation of free, monomeric AHR within cells [97]. In response to a molecular trigger, AHR dissociates from the core complex and localizes to the nucleus where it heterodimerizes with its binding partner to activate gene expression. At present, the signals regulating nuclear translocation are still being elucidated; however, there appears to be at least two separate mechanisms depending on whether the receptor is activated by ligand or an endogenous signal.

HSP90 has an important role in maintaining AHR stability and structure, with the central region binding to both the bHLH and PAS domains of AHR. HSP90 appears to be essential for maintaining AHR in its ligand-binding conformation, with AHR losing its dioxin-induced activity when HSP90 is disrupted *in vitro* [100–102]. Ligand binding appears to disrupt the binding of HSP90 to the PAS domain, which is adjacent to or just within the LBD, but not to the bHLH region, resulting in a conformational change that retains HSP90 binding during nuclear translocation. To explain this phenomenon, McGuire *et al*. [101] postulate that after ligand binding, HSP90 promotes a conformational change to accommodate the binding by AHR's dimerization partner, ARNT, following translocation to the nucleus, and therefore remains bound to AHR through the bHLH domain during nuclear shuttling. XAP2 is a 38-kDa protein with

significant homology to the immunophilins, such as FKBP52 [99]. Although not absolutely required for the structural integrity of the cytoplasmic core complex, XAP2 does appear to have a significant role in maintaining cytoplasmic localization [103]. In mice, nuclear localization appears to rely on importin β, which recognizes the bipartite NLS found in AHR. In the cytoplasm, XAP2 binding in the core complex masks the NLS, preventing importin binding and subsequent nuclear localization [103]. Ligand binding induces a conformational change in XAP2, resulting in dissociation of XAP2 and HSP90 from AHR. This unmasks the bipartite NLS, making it available for importin binding and thus nuclear import [98,104]. However, XAP2 does not seem to fulfill the same role in humans, as it appears to shuttle into the nucleus still bound to AHR [105,106]. This mechanism also involves PKC-mediated phosphorylation of two serine residues adjacent to the NLS—Ser12 and Ser36—which inhibits nuclear translocation by masking the NLS from importins [107].

Although ligand-dependent nuclear translocation seems to rely on ligand-induced conformational change of XAP2 and HSP90, evidence for another pathway involving cyclic-nucleotide-dependent signaling has been reported [97,98,108,109]. cAMP has been shown to interact with the AHR core complex and induce nuclear translocation [109]. However, the conformational change induced by this interaction reduced the ability of AHR to dimerize with ARNT, changed the DNA recognition sequence of AHR, and reduced dioxin-induced target expression [98,109].

Following nuclear translocation, it has been reported that phosphorylation of AHR and ARNT is essential for heterodimerization and thus DNA-binding activity [22]. The kinases involved were PKC and members of the ERK/MEK signaling pathway. This once again illustrates the importance of cooperative control with endogenous signaling pathways in xenobiotic metabolism. A novel nuclear binding partner has also been identified, which acts through the cAMP-related signaling pathway: the RelB subunit of NF-κB [110–112]. This heterodimer behaves in a PKA phosphorylation-dependent manner, with AHR DNA binding identified in the promoter region of interleukin-8. Although this alternative binding partner has a role in coregulation of inflammatory genes, acting in concert with the NF-κB pathway, any possible role in P450 regulation has yet to be investigated.

In summary, AHR is a promiscuous receptor that has a key role in metabolic response to environmental and dietary toxins. The mechanisms regulating AHR-induced P450 regulation are complex, requiring cross talk between many signaling and physiological processes. It is worthy to note that the majority of AHR research to date has been conducted *in vitro*, especially using murine cell lines. This has a number of limitations, including, in particular, AHR function is linked with a number of physiological processes, therefore important interactions will be missed and there are significant species differences in the structure and function of AHR. The application of AHR-humanized mice could be of use to further establish the *in vivo* regulation of this signaling pathway [113].

8.2.4 Receptor Cross Talk

In order to maintain a robust response to chemical challenge, multiple transcription factors have evolved to modulate P450 expression. This has created a complex interacting network of gene regulation [114]. The cross talk between the transcription factors, both in the ligands with which they interact and the downstream genes that they regulate,

allows the systems to be fine-tuned for the detoxification of specific compounds and the interactions between endogenous and exogenous pathways to be controlled. It is, therefore, imperative to understand the degree to which this phenomenon controls both *in vitro* and *in vivo* drug-metabolizing enzymes. This section discusses the cross talk between the three main transcription factors and the regulation of certain key P450s involved in drug metabolism.

8.2.4.1 CYP1A1/2. The CYP1A enzymes are associated with the metabolism of environmental toxins and aromatic hydrocarbons, providing protection against acute dioxin-induced hepatocellular necrosis and hepatic inflammation [4,5,115,116]. They are primarily under the control of AHR, with *Cyp1a1* and *Cyp1a2* possessing conserved XRE clusters in their proximal promoters [116]. However, other response elements have been located in the promoters of these genes, suggesting regulatory roles for other transcription factors. Also, PXR ligands can induce AHR target genes through the transcriptional regulation of AHR [4]. Yoshinari *et al*. [5] recently provided evidence that binding of the hCAR/RXRα heterodimer to a conserved ER8 motif within the proximal promoter of the CYP1A1 and CYP1A2 genes increases their expression. This finding suggests that drugs that activate CAR could alter the metabolism of CYP1A1 and CYP1A2 substrates and therefore be a source of drug–drug interactions. Glucocorticoid response elements (GREs) have also been identified in the promoter region of the rat *CYP1A1* gene, which, once activated, interact with the initiation complex to enhance AHR-induced transcription [54]. However, regulation by this mechanism appears to be species specific and does not occur in humans.

Inflammation has been reported to negatively regulate CYP1A1 expression, with exposure to UV-B repressing the induction of CYP1A1 immediately following irradiation, although CYP1A1 is induced in an AHR-dependent manner several hours following irradiation [115,117]. This was attributed to direct interaction between AHR and the NFκB RelA (p65) subunit, interfering with cofactor recruitment to the promoter. The repression of Cyp1a1 expression in response to oxidative stress had been reported by Morel and Barouki, who identified nuclear factor 1 (NF1) as the mediator of this effect [118]. Reciprocal cross talk between AHR and Nrf2 has also been recently reported, with XRE sequences being located in the Nrf2 promoter [12], and ARE sequences in the AHR promoter [119]. It has been suggested that a functional protein interaction may also exist between these molecules, although no data is currently available [120]. The reciprocal cross talk between these two systems further highlights the key role of AHR in the protection of the body from damage caused by electrophilic metabolites.

Regulation of CYP1A expression is therefore controlled by a number of core interacting transcription factors. However, because of the many endogenous roles played by the AHR, they are also subject to regulatory control by diverse signaling pathways, including those involved with inflammation and oxidative stress response, playing a key role in protecting the body from xenobiotic challenge.

8.2.4.2 CYP2B6. CYP2B6 metabolizes a wide range of drugs, including cyclophosphamide, valproic acid, ketamine, aminopyrine, methadone, and bupropion [59]. Its overall role in drug metabolism in man, however, has been considered minor [59,121]. CYP2B6 is induced by activators of hCAR, with the activated hCAR/RXRα heterodimer binding to the DR4 motif in the phenobarbital-responsive-element module

(PBREM) in the promoter [54,59]. However, cross talk with human pregnane X receptor (hPXR) has also been implicated in CYP2B6 regulation, through binding to the DR4 motif, all be it with lower affinity. A response element termed the *distal xenobiotic responsive enhancer module* (XREM) has also been identified as activated by either hCAR/RXRα or hPXR/RXRα [122]. This element works synergistically with the activated PBREM [59,122].

Although cross talk with PXR is reported to be part of the mechanism in the control of CYP2B6, other receptors are also implicated. For instance, in rodents, the glucocorticoid receptor (GR) directly activates CYP2B/Cyp2b expression [123,124]. Although the GR does not directly activate CYP2B6 in humans, it can affect CYP2B6 expression indirectly through induction of CAR and PXR [59]. Recent studies from the Negishi group have suggested that for maximal activity, the CYP2B6 promoter can be synergistically activated by the PBREM and a novel 52-bp response element, known as the *okadaic acid response element* ($OARE_{KI}$) (Fig. 8.2) [125]. The $OARE_{KI}$ contains a DR1 motif, which possesses an HNF4α binding site, a CACCC motif, which binds early growth response 1 (EGR1) protein, and an E box motif. EGR1 binding is required for maximal CAR-induced CYP2B6 activity, facilitating CAR/HNF4α cross talk by inducing DNA looping to bring the proximal $OARE_{KI}$ into proximity with the distal PBREM [126]. A recent study has demonstrated that the CCAAT/enhancer binding protein α (C/EBPα) and HNF4α cooperate with CAR to control CYP2B6 expression [121]. HNF4α binding to a proximal element located in the $OARE_{KI}$ (−217 bp) favors

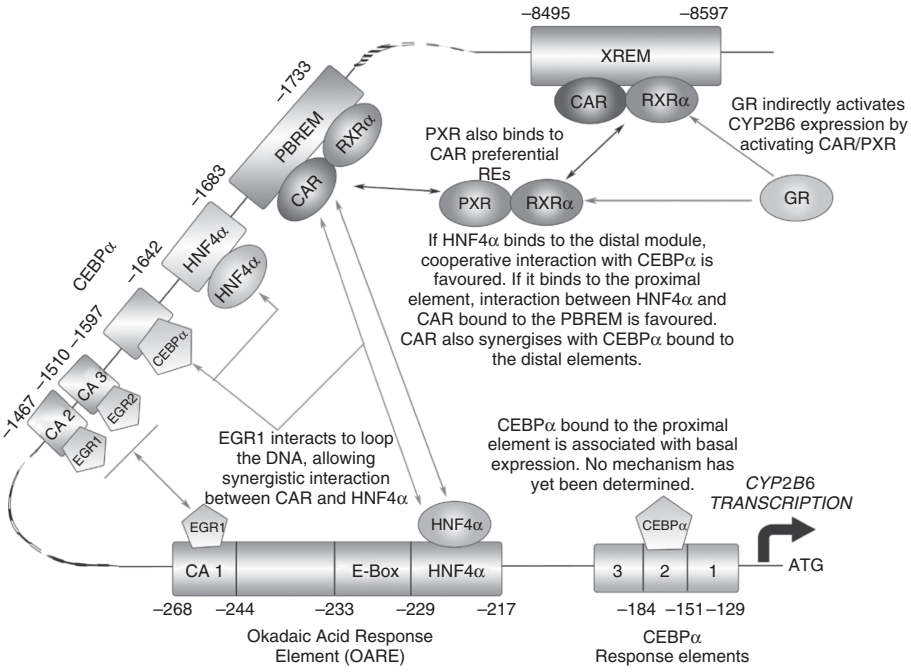

Figure 8.2 Regulatory network controlling CYP2B6 expression. *Source*: Adapted from Benet *et al*. [121], with additional information from Inoue and Negishi [126] and Wang and Tompkins [59]. (See color insert.)

CAR interaction, whereas binding to the distal region (−1642 bp) favors interaction with C/EBPα (Fig. 8.2). Variation in expression and binding of HNF4α and C/EBPα to the CYP2B6 promoter has been correlated to interindividual variations in CYP2B6 expression and activity, suggesting their importance in the regulation of this gene [121]. It is clear that CAR-induced transcriptional regulation of CYP2B6 involves intricate cross talk between CAR, HNF4α, EGR1, and C/EBPα as a minimum. However, the role of PXR in this mechanism remains to be clarified.

8.2.4.3 CYP2C8. *CYP2C8* is the most inducible gene in the CYP2C family, with a role in the metabolism of drugs such as rosiglitazone, paclitaxel, cerivastatin, and chloroquine, in addition to endogenous compounds, such as arachidonic acid [9]. The regulation of this protein remains poorly characterized, although DR4 binding at −8.8 kb is needed for CAR and PXR-mediated CYP2C8 induction [9]. Both CAR and PXR can bind to this element in response to ligands. However, Ferguson *et al*. [9] found that this response is not apparent when the reporter gene assay is performed in the HepG2 cell line but is seen when using metabolically competent primary human hepatocytes, demonstrating the limitations of HepG2 cells for drug induction studies. As with CYP2B6, basal expression of CYP2C8 involves HNF4α binding to a DR1 motif in the basal promoter region. A second functional HNF4α binding site has also been identified in the proximal promoter region [127]. Site-directed mutagenesis of the CYP2C8 promoter has shown that both HNF4α sites are important for RIF-induced PXR-mediated expression of CYP2C8, with that in the proximal promoter region being essential. Also, silencing of HNF4α using RNA interference (RNAi) decreased basal CYP2C8 reporter activity by 48% and abolished RIF-mediated induction. However, this treatment also repressed expression of CAR and PXR by 60% and 40%, respectively [127]. In light of the current limited information, further studies on the role of CAR in CYP2C8 control are required.

In addition to the above regulatory pathways, the GR also appears to be involved in the induction of members of the CYP2C gene family, with a functional GRE being located in the proximal promoter region in many CYP2C genes [9,54]. Ferguson *et al*. [9] demonstrated that CYP2C8 induction by the prototypical GR activator, dexamethasone, occurs by direct interaction with the GRE located in the promoter region of the gene, and not by interaction with PXR or CAR. A recent study in HepG2 cells identified retinoic-acid-receptor-related orphan receptor (ROR) response elements (ROREs) in the promoter of CYP2C8, which are activated by RORα1, RORα4, and RORγ1 isoforms [128]. The importance of RORs in the control of CYP2C8 was further supported by the finding that knockdown of the three isoforms resulted in a 50% decrease in *CYP2C8* mRNA. Since natural agonists of these receptors include cholesterol and its metabolites, CYP2C8 expression is likely to be influenced by normal physiological processes. These receptors are also linked to the control of circadian rhythm and therefore could be associated with circadian expression of the CYP2C genes. In summary, the regulatory control of CYP2C8 is still poorly understood, although certain key factors have been identified. More research is therefore required to integrate these signals into an understandable network and to more fully characterize regulation by CAR and PXR.

8.2.4.4 CYP2C9. CYP2C9 is the most highly expressed of the CYP2C family [129], with identified substrates including tolbutamide, diclofenac, and *S*-warfarin [130]. In

human hepatocytes, CYP2C9 could be regulated with prototypical PXR, CAR, and GR activators (RIF, PB, and dexamethasone, respectively), indicating that all three receptors have a role in the regulation of this enzyme [131]. Subsequent studies suggested that CAR plays a role in the basal expression of CYP2C9, with constitutive expression in HepG2 cells increasing with stable transfection of mouse or hCAR [131,132]. Two DR5 response elements, in a configuration similar to that of the PBREM and XREM modules, are located in the proximal promoter at −2899 bp and preferentially bind CAR over PXR [132]. A DR4 CAR/PXR binding motif at −1839 bp has also been identified [131]. Mutagenesis of these binding sites provided evidence that both are sensitive to CAR activation [132]. The induction of CYP2C9 by treatment with RIF, hyperforin, and PB has been shown to be mediated by hPXR binding to the DR4 response element [10,133]. The proximal DR4 binding site is therefore essential for activation, with the distal site acting in a cooperative capacity.

HNF4α plays an essential central role in the regulation of CYP2C9, with HNF4α binding sites located at −211, −185, and −152 bp. The site at −185 bp plays a central role in HNF4α-induced CYP2C9 expression and CAR/PXR synergism [127,134]. The HNF4α binding sites cooperate with the CAR/PXR responsive DR4 motif for maximal gene expression [127]. The underlying mechanism appears to be as a result of recruitment of a number of coactivators to the promoter, including cAMP response element binding protein (CBP), PGC-1α, PRIP-interacting protein with interacting methyltransferase (PIMT) domain, and nuclear receptor coactivator 6 (NCOA6) [135]. Current evidence suggests that NCOA6 forms a bridge between CAR and HNF4α bound to their respective response elements by direct interaction with the LXXLL motifs of each. NCOA6 therefore acts as a mediator promoting synergistic cross talk between CAR and HNF4α. It is not yet clear whether the same mechanism applies to hPXR-mediated control.

Other pathways that do not appear to directly interact with CAR/PXR-induced CYP2C9 expression have also been detailed. An imperfect GRE sequence at −1675 bp allows direct activation of the promoter by the GR, which appears to act synergistically with CAR/PXR [132]. HNF3γ, also known as FOXA3, has been implicated in the regulation of CYP2C genes, being found to bind to HNF3γ response elements in the promoter region, although a mechanism has yet to be elucidated [136]. A study by Drocourt *et al*. [137] suggested that the VDR can also regulate CYP2C9 expression. However, they point out that the concentrations of 1α,25-dihydroxyvitamin D_3 required for this interaction are above the levels seen physiologically, and therefore, the relevance of this interaction is questionable. A recent study has also shown the transcription factors GATA-2, -4, and -6 induce CYP2C9 through direct interaction with response elements in the promoter [138]. Again, any potential cross talk with other motifs has yet to be investigated, but the tissue-specific nature of GATA transcription factors could make this an interesting pathway when considering gene expression in extrahepatic tissues.

The regulation of CYP2C9 appears unusual in comparison to the other commonly inducible P450s. Although, HNF4α is again central to promoter activation, regulation by CAR and PXR is such that constitutive expression appears to be mediated through a CAR-mediated pathway, whereas ligand induction occurs through a PXR-mediated pathway, showing a more physiologically cooperative cross talk between these receptors than is often seen. Cross talk mediated through cofactors is also apparent in the mechanism underlying cooperation of CAR and HNF4α through mediation by NCOA6.

8.2.4.5 CYP3A4. CYP3A4 is the major P450 expressed in human liver and is responsible for the metabolism of approximately 60% of current drugs. The variation in CYP3A4 expression due to enzyme induction is therefore a major determinant of drug–drug interactions [39]. The key regulator of CYP3A4 expression is PXR, with the hPXR/RXRα heterodimer binding to an ER6 PXRE motif located at −172 bp relative to the transcription start site in the proximal promoter region [139–141]. Mutation of this element led to a 50% reduction in RIF-mediated response, indicating that this motif acts cooperatively with other regulatory elements [140]. A distal XREM enhancer has been identified at approximately −7800 bp, consisting of three nuclear-receptor-binding sites, dNR1-3. Disruption of the PXRE in the proximal promoter and the dNR1 imperfect DR3 binding motif in the distal XREM represses xenobiotic response by approximately 85%, indicating that both elements are essential for CYP3A4 expression. The dNR3 site is also required for maximal gene expression [140]. A further polymorphic enhancer module, known as the *constitutive liver enhancer module of CYP3A4* (CLEM4), has been identified at −11.4 kbp [142]. This is also required for maximal gene expression. This module contains multiple cis-acting elements, including binding sites for HNF-1, USF-1, AP-1, and HNF4α, as well as a perfect ER6 PXR binding motif. This enhancer region acts synergistically with the distal XREM and proximal promoter in PXR-mediated *CYP3A4* induction [143]. This region also appears to be involved in constitutive CYP3A4 expression [142]. However, the motif located in this region has been found to be significantly less competitive for PXR/RXRα binding relative to motifs found in the distal XREM and proximal promoter, instead the motif has a higher affinity for a PXR homodimer *in vitro* [143]. This homodimer has yet to be described *in vivo*, but potentially, this could result in a previously uncharted regulatory mechanism associated with CYP3A4 control.

All PXR binding motifs described above have also been shown to bind hCAR, although binding to the proximal promoter region alone is insufficient for CAR-induced expression. Therefore, evidence suggests that CYP3A4 is coordinately regulated by hCAR and hPXR [144,145]. Although PXR/CAR cooperation appears to be the main regulatory pathway connected to ligand-induced control of CYP3A4, neither are essential for constitutive expression, with individual disruption of these genes *in vivo* having no influence on basal CYP3A4 expression [142]. Istrate *et al*. [146] have used gel shift and LS174T-based reporter gene assays to show that a number of transcription factors can bind the ER6 and DR3 motifs, thus decreasing PXR-mediated gene induction through competition. For instance, the thyroid receptor (TR) α1 can bind to the response elements to repress CYP3A4 basal and ligand-induced expression. The ratio of PXR:TRα1 was moreover found to be correlated to basal expression level, suggesting a role in endogenous control. The VDR has also been found to compete with PXR for ER6, DR3, and DR4 binding sites to induce CYP3A4 expression in rat and human liver and intestinal slices, all with lower activity than PXR [137,147]. However, a recent study has shown that VDR and PXR can act synergistically in intestinal cell lines, apparently through VDR binding to the PXRE located in CLEM4 [148].

HNF4α also has a significant role in controlling CYP3A4 expression. Tirona *et al*. have identified a DR1, HNF4α binding site immediately upstream of the dNR1 and dNR2 binding sites in the XREM (−7783 bp). This was shown to be essential for ligand-induced promoter activity. *In vitro* reporter transactivation assays in HepG2, Caco-2 and HeLa cell lines indicated that basal and induced promoter activities are mediated through PXR/CAR, with the activity increasing synergistically in the presence of

HNF4α [149]. In contrast, the PXRE located in CLEM4 is flanked by two downstream HNF4α binding motifs [143], which suppress rather than facilitate PXR/CAR-induced gene expression as a consequence of HNF4α interference with PXR DNA binding. Liu *et al*. also reported that while disruption of the ER6 motif in the CLEM4 domain has no effect on basal expression, disruption of the DR3 motif in the XREM results in a decrease in basal activity. Other regulators of CYP3A4 expression have also been suggested. These include STAT1, HNF1, HNF3β (FOXA2), HNF3γ (FOXA3), and C/EBPα [150]. A systematic *in vitro* analysis in HepG2 and HuH7 cell lines has been performed by Bombail *et al*., in which putative binding sites were disrupted to identify functional interactions [151]. This study identified C/EBPα as a regulator of CYP3A4 expression, with mutation of the C/EBPα binding site at −132 bp causing a 60% decrease in basal expression and a decrease in I_{max} associated with RIF-, PB-, and metyrapone-mediated activation. In a previous work, two distal C/EBPα sites (located at −1402 and −1668 bp) were identified in the activation of CYP3A4 expression. HNF3γ is also a putative regulator, with a binding site located at −1730 bp. However, this receptor does not activate CYP3A4, but rather synergizes with C/EBPα, promoting gene expression by chromatin remodeling [151,152]. In addition to these findings, Bombail *et al*. reported that disruption of a putative Sp1 site at −104 to −97 bp resulted in a 50% decrease in promoter activity following PB treatment. Despite the presence of a putative GRE in the proximal promoter region, it is likely that GR agonists influence CYP3A4 expression by inducing PXR expression rather than interacting directly with the promoter [147,153]. CYP3A4 expression is also influenced by various physiological processes, as demonstrated by its regulation by FOXA2 and FOXA3, which have numerous endogenous roles, including embryonic development, organogenesis, and glucose homeostasis [154]. Other endogenous modulators include the HIF-1α transcription factor, which is upregulated in response to oxidative stress, and interacts to downregulate drug-metabolizing enzymes, including CYP3A4, in order to prevent the production of reactive oxygen species [155]. This seems to act through an indirect mechanism, such as downregulation of PXR and CAR, rather than through direct promoter binding. It is also important to remember that these transcription factors are subject to control by endogenous signaling molecules. This is highlighted by a recent report linking CYP3A4 expression to the cell cycle via phosphorylation of PXR by cyclin-dependent kinase 2 [156].

As this section has shown, although each of the enzymes discussed is preferentially controlled by a given transcription factor or nuclear receptor, a large variety of signaling pathways and coregulating molecules interact to control gene expression. The redundancy inherent in this system could be explained by the need to adapt to a wide range of environmental demands and endogenous stimuli. Our understanding of the interactions underlying transcriptional control is rapidly increasing. However, the networks we have currently identified are simplistic, omitting key endogenous signaling information that can have dramatic consequences for drug metabolism. While this section has aimed to give a broad overview of interaction at the level of P450 promoter regulation, it has of necessity excluded another level of functional cross talk, which exists between different nuclear receptors and transcription factors. Several reviews are now available, which discuss functional cross talk between nuclear receptors and certain transcription factors [15,16,114]. This interaction can significantly affect downstream regulation of target genes and thus the overall P450 expression profile.

8.3 OTHER CONSIDERATIONS FOR TRANSCRIPTIONAL REGULATION OF CYTOCHROME P450s

8.3.1 Methodological Challenges

Understanding P450 regulation is a vital step toward understanding drug interactions. Although the mechanisms underlying P450 transcriptional regulation are becoming clearer, methodological difficulties have introduced other issues that must be considered when analyzing current evidence.

Although *in vitro* methods, such as reporter gene assays, have been used to identify P450-inducing agents and mechanisms of transcriptional control, cell-based systems are unable to fully model transcriptional activation. Traditional overexpression systems have limitations because PXR and CAR will spontaneously translocate to the nucleus, probably because the members of the cytoplasmic retention complex become saturated [28,157]. This removes one of the key mechanisms underlying transcriptional regulation. A further problem is that with the exception of primary hepatocytes and a handful of immortalized cell lines, such as the HepaRG model [158–160], the majority of cell lines do not express physiologically relevant levels of both the P450-related transcription factors or P450s themselves. Cells also have the disadvantage that they cannot model systemic interactions, a significant problem when transcriptional regulators are influenced by systemic cues, such as hormonal signaling and energy state.

The use of conventional animal models to study ligand induction of P450s is therefore common, being able to model induction in a more physiologically relevant manner. However, because of the divergence of the metabolic system between species, models do not accurately predict P450 induction in humans [161]. The consequences of this can be profound. For instance, from a pharmaceutical perspective, failing to consider potential differences in drug metabolism could result in drugs reaching clinical trials that are therapeutically ineffective or, much more importantly, toxic to humans [162]. Species specificity is therefore a major issue to be addressed when considering results from animal models.

8.3.2 Species Specificity

The mechanisms underlying species specificity in drug induction are complex. However, species differences in P450 induction can often be ascribed to differences in the amino acid sequence of the LBD of the transcription factor involved [7,163–168]. Changes in the activity of NLS sequences have also been associated with alterations in nuclear localization, resulting in aberrant gene transcription [92,105,106,169,170]. Lichti-Kaiser *et al*. [37] have also identified species specificity in the response of hPXR and mPXR (mouse pregnane X receptor) to the cAMP nucleotide signaling pathway. CYP3A4 expression was found to be synergistically increased on treatment with RIF and the PKA inhibitor H89, whereas it decreased following treatment with H89 and pregnenolone-16α-carbonitrile (PCN). However, the study compared data from HepG2 cells transduced with an adenoviral expression vector containing hPXR with that from primary mouse hepatocytes, which may influence the findings. In the case of AHR, species-specific differences in receptor protein stability (hAHR< mAHR) and histone deacetylase complex recruitment have been implicated in differential response [170]. Improved understanding of species differences in nucleocytoplasmic shuttling,

TABLE 8.4 Examples of Species-Specific Ligands for PXR and CAR

Drug	Interacts with	Species			
		Mouse EC_{50} (nM)	Action	Human EC_{50} (nM)	Action
PCN	PXR	200–700	+	>10,000	N/A
Rifampicin		Weak		200–3000	+
SR12813		4100	+	120–200	+
Hyperforin		NR		23	+
5β-Cholestan-3α,7α, 12α-triol		2500	+	5000	+
TCPOBOP	CAR	20–100	+	Weak	N/A
CITCO		Weak		25–304	+
5α-Androstan-3α-ol		250–1500	–	1000–>10,000	–
Meclizine		25	+	500–1000	–
5β-Pregnane-3,20-dione		670–3000	+	>10,000 ≫10,000	Weak+ –
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	AHR	0.6	+	0.4–9	+

Includes EC_{50} (nM) where data is available.
NR, not reported; +, agonist; –, antagonist (inverse agonist, CAR only).
PXR and CAR data adapted from Refs 15 and 16. AHR data adapted from [177] (mouse), [178] (human), and [179].

cell signaling pathways and subsequent activation mechanisms is therefore of central importance. The availability of mouse models humanized for both the receptors which mediate drug induction as well as the P450's involved can help circumvent many of these problems.

Although the precise mechanisms have yet to be fully elucidated, numerous P450 inducers have been identified which interact with transcription factors in a species-dependent manner. A selection of these ligands has been detailed in Table 8.4. These include numerous drugs, endogenous compounds, environmental contaminants and certain herbal medicines. Most of this data has been generated using *in vitro* reporter gene assays, which are a useful model for identifying species-dependent ligand effects on transactivation activity of transcription factors. However, there are certain ligands that have proved of particular importance in the study of receptor activation. The macrolide antibiotic RIF and PCN have been shown to be species-specific activators of PXR, with RIF activating hPXR and PCN activating mPXR [15,171–173]. In the same way, 6-(4-chlorophenyl)imidazo-[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO) and 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) are species-specific activators of CAR, with CITCO preferentially activating hCAR and TCPOBOP activating mCAR [15,171,174]. All four compounds are high affinity ligands for their preferred receptor, showing significantly lower or no affinity for the other species receptor. They are therefore commonly used as prototypical inducers of CYP3A4 (hPXR), Cyp3a11 (mPXR), CYP2B6 (hCAR), and cyp2b10 (mCAR), respectively, to validate models of P450 transactivation. With regard to AHR, a study by Westerink *et al*. identified differential CYP1A inducers between rat and human cell lines [175], while Boutros *et al*. also suggested that TCDD, although not affecting core AHR genes such as the CYP1 family, does exert species-specific effects, having a binding affinity for hAHR 10-fold less than that of mAHR, which could significantly

affect downstream regulation of target genes [176]. In view of the marked differences that can exist between ligand-induced regulation of drug metabolism, understanding how emerging drugs interact with human receptors before clinical testing is vital.

In order to address this issue, a number of new animal models have been created, which are humanized for the metabolic enzymes and transcription factors involved in drug metabolism [174,180,181]. Several approaches have been used in the construction of such models. Katoh *et al*. [181] created chimeric mice in which the innate hepatocytes of an immunocompromised mouse (uPA$^{+/+}$/SCID) were replaced with >90% human hepatocytes *in vivo*, resulting in an artificially humanized liver. However, the drawback of this model is that because these models must be produced as required, it requires a ready supply of human hepatocytes, which are both costly and hard to source as well as having the innate interindividual variability seen in the human population. The technique may also be restricted under legislation regulating the use of animals in experiments in some countries. A more common approach is to introduce the human gene into the mouse genome, either under the control of its own promoter by random integration or by replacing the endogenous mouse gene [174,180,182]. This has the advantage that the mouse lines can be continuously bred as well as potentially crossed to other mouse lines carrying linked genes of interest. This potentially expands the number of criteria that can be studied in a single model and helps overcomei potential ethical and sourcing issues encountered with the chimeric models.

Many humanized models are now available, including a range of humanized P450 models, such as CYP2D6 and CYP3A4, to enable drug metabolism to be characterized. However, the key models from the perspective of P450 transcriptional regulation are those in which the transcription factors are humanized. Some of these models are reviewed by Stanley *et al*. [16] and Gonzalez and Shah [180]. A battery of PXR and CAR models have now been developed to allow PXR- and CAR-mediated P450 induction to be dissected [174,183,184]. These include a model in which both PXR and CAR have been replaced with the human genes under the control of the corresponding endogenous promoters, as well as models in which one receptor is humanized while the other is deleted. Together with more traditional single humanized and knockout models, these models have the potential to allow species specificity and the role of receptor cross talk in P450 induction to be fully analyzed. A further advantage of these models is that their design allows expression of PXR and CAR splice variants at physiologically relevant levels, allowing interindividual variability to be studied [183] (Section 8.8.3). These models therefore provide a powerful tool for use in the analysis of mechanisms underlying species-specific regulation of P450s. Although not perfect, with receptors still under the influence of the mouse physiological environment, the data that can be gained from these humanized models is more relevant to humans than traditional animal models, especially since the majority of species-specific activity is thought to depend on the sequence of the LBD. As these models become more complex, with multiple genes being humanized in one model, data quality is likely to improve.

8.3.3 Splice Variants

Alternative splicing of mRNA is ubiquitous in eukaryotes, and many human genes are subject to the process, with 86% having a minor isoform frequency of 15% or greater, resulting in the production of different mRNAs, thus potentially generating multiple proteins from a single gene and greatly increasing genomic diversity [185]. There are

a number of different mechanisms involved in alternative splicing of mRNA, including exon skipping, intron retention, and the use of alternative splice donor and acceptor sites [186,187].

Alternative splicing of human P450 mRNAs was first described in the 1980s [188]. However, the identification of splice variants of hCAR and hPXR was more recent. Different-sized hPXR mRNAs were first identified in the late 1990s [189,190], with Fukuen *et al*. describing a total of 9 hPXR SVs whose expression levels varied significantly across a panel of 15 liver samples [191]. Further work to define the expression of hPXR SVs found the most common variants to be PXR.2 (lacking 111 nt) and PXR.3 (lacking 123 nt), representing approximately 6.7% and 0.32%, respectively, of hPXR mRNA in human liver and also found in a number of other tissues, including brain, heart, colon, and bone marrow [3]. PXR.2 was subsequently reported not to function in a transactivation assay because of the failure of ligands to bind to the LBD in a productive manner, probably as a result of a change in protein structure and the continued binding of corepressors [192].

Choi *et al*. in describing murine CAR, also reported the existence of a variant mRNA (mCAR2), which lacked exon 8 and was unable to function in a transactivation assay [45]. The first SVs of the hCAR gene were reported in 2003 when Savkur *et al*. described an mRNA for hCAR with a 4-amino-acid insert between exons 6 and 7 and a 5-amino-acid insert between exons 7 and 8 resulting from the use of alternative splice acceptor sites, and another with a complete deletion of exon 7 and loss of 39 amino acids [193]. In addition to these variants, Auerbach *et al*. [194] reported further hCAR SVs with one or other of the insertions between exons 6/7 and 7/8 and confirmed that such variants had compromised function. The range of hCAR SVs was extended dramatically when Lamba *et al*. reported 22 unique mRNAs from a panel of human liver and other tissues [195]. Several of these SVs had premature termination codons and failed to produce protein, but many had significant deletions or insertions, yielding proteins significantly different in size from the wild-type, or reference, form and which had altered N- and/or C-terminal amino acids. The hCAR SVs were differentially expressed, with only the hCAR reference form found in the intestine, whereas spleen, heart, and prostate expressed only the SVs, and were nonfunctional in transactivation assays, although the authors speculated that such SVs might possess biological function(s) yet to be identified [195]. A biological function was demonstrated for the hCAR SV with a 5-amino-acid insertion between exons 7 and 8, termed CAR3 by Auerbach *et al*., who found activity in a transactivation assay with an optimized 3× DR4 reporter using CITCO, thus defining CAR3 as a ligand-activated receptor in contrast to the reference form (CAR1) [196]. The same group further demonstrated that CAR2, with a 4-amino-acid insertion between exons 6 and 7, displayed a regulatory response distinct from that of CAR1, speculating that the ligand-binding pocket may be modified by the additional residues found in CAR2 [197]. Subsequently, CAR2 was also shown to be a ligand-activated receptor present in approximately 30% of total CAR transcripts in human hepatocytes and uniquely responsive to the plasticizer di(2-ethylhexyl) phthalate (DEHP) [198]. These latter findings—that a nuclear receptor present at significant levels in human liver (but absent in other animal models) is highly responsive to a chemical agent commonly found in a wide range of everyday materials, including medical devices—have potentially significant implications for safety testing in general and predictive human toxicity in particular.

To address these concerns, a unique panel of transgenic mice humanized for CAR and/or PXR have been generated, which are nulled for murine CAR and PXR and express the reference forms and two major SVs of hCAR (CAR2 and CAR3) and hPXR (PXR2 and PXR3) under the control of the endogenous mouse promotor [174]. Expression levels of the CAR and PXR SVs in these mouse models were comparable to that found in a panel of human livers, with the exception of hPXR2 in which the expression was approximately fourfold higher in the transgenic mouse [183]. The expression profile of the PXR SVs in the hPXR mouse found that PXR1 was expressed in a range of tissues at varying levels—liver > small intestine > kidney > lung > gonads > brain—while PXR2 was expressed, as a % of total PXR mRNA, at approximately the same level in all tissues, and PXR3 at highest levels in the kidney, gonads, and brain (Bower, CCM, unpublished observations). These mouse models provide a useful tool to investigate the role of CAR and PXR in drug metabolism, disposition, and efficacy in humans.

8.3.4 Genetic Polymorphisms

Interindividual variation in human drug metabolism is a significant cause of variability in drug response [199]; a main contributor is genetic polymorphism [200]. P450s are a highly polymorphic group of enzymes, with more than 350 different functional alleles so far identified in the genes, accounting for 40–50% of P450-dependent metabolism in humans [201,202]. Most of the characterized polymorphisms are variations in the coding sequence, such as SNPs, deletions, or gene amplifications [203,204]. They can, however, also be in gene promoters or intronic regions, which interfere with transcription factor binding or cause alternative or aberrant mRNA splicing [202,204]. Many of these polymorphisms manifest themselves as enzymes possessing altered activity [201]. One example of this is seen in the *CYP2B6*6* polymorphic isoform, which possesses two SNPs, 516Gly > Thr and 785Ala > Gly [59]. These substitutions result in alternative gene splicing, yielding a product with a deletion of exons 4–6 and that displays a significant reduction in catalytic activity and protein expression relative to the reference *CYP2B6*1*. Presence of these polymorphic variants can therefore significantly affect downstream drug metabolism.

Another source of interindividual variability in P450 expression is the polymorphisms in their regulators. One such polymorphic regulator is PXR, with 373 SNPs having been identified, and many of these proving to be functional [205,206]. As an example of the effect of PXR polymorphism on downstream gene expression, a study by Wang *et al*. [205] has examined the effect of two haplotypes of PXR commonly found in the Han Chinese population, H1 (TCAGGGGCCACC) and H2 (CCGAAAAC-TAAT), on P450 expression, using CYP3A4 activity as a marker. They found that those with the haplotype pair H1/H1 had much higher inducible CYP3A4 metabolic activity than those subjects with the H1/H2 and H2/H2 pairings following treatment with St Johns' Wort, as indicated by the significant differences in AUC_{0-t} and $AUC_{0-\infty}$ for the CYP3A4 probe drug nifedipine and its metabolite, dehydronifedipine. This phenomenon has been further confirmed by identification of functional polymorphisms in the FOXA2 transcription factor, which affect *CYP3A4* transcription and are also linked to diseases, such as type II diabetes [207]. Polymorphisms in P450s and the transcription factors, which control their expression, can therefore play a significant role in interindividual variation in xenobiotic metabolism. Understanding the genetic differences in P450 regulation remains an important subject for future study.

8.4 CONCLUSION

The adaptive response system that has been evolved by organisms to protect the cell from the many forms of toxic insult plays a central role in the metabolism and disposition of drugs. The importance of this system is underlined by its robust nature and built-in functional redundancy that allows at least a partial response in the absence or failure of a component part. The species differences observed in the regulation of P450 expression underline the importance of developing relevant models to test for human drug safety and efficacy, and in which factors such as gender, circadian rhythm, diet may also be taken into account. It is thus clear that an understanding of how drug metabolism is regulated, and the multitude of variable factors involved, is crucial to the optimal application of drug treatments and minimization of unwanted side effects and drug–drug interactions.

ABBREVIATIONS

AF-2	Activation Function 2
AHR	Aryl Hydrocarbon Receptor
AP-1	Activator Protein 1
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
bHLH	Basic Helix-Loop-Helix
bp	Base Pair
cAMP	cyclic AMP
CAR	Constitutive Androstane Receptor
CCRP	Cytoplasmic CAR Retention Protein
Cdk2	Cyclin-Dependent Kinase 2
C/EBPα	CCAAT/Enhancer Binding Protein α
ChIP	Chromatin Immunoprecipitation
CHIP	C-terminal of HSP70 interacting protein
CITCO	6-(4-Chlorophenyl)imidazo[2,1-*b*]thiazole-5-carbaldehyde *O*-(3, 4-dichlorobenzyl)oxime
CLEM4	Constitutive Liver Enhancer Module of CYP3A4
CREB	cAMP Response Element Binding Protein
CYP	Cytochrome P450
DBP	Albumin D-Box Binding Protein
DEHP	Di(2-ethylhexyl) Phthalate
DR	Direct Repeat
EGR1	Early Growth Response 1
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Elements
HIF1α	Hypoxia Inducible Factor 1α
HLF	Hepatic Leukemia Factor
HNF1	Hepatic Nuclear Factor 1
HNF3β	Hepatic Nuclear Factor 3β
HNF3γ	Hepatic Nuclear Factor 3γ
HNF4α	Hepatic Nuclear Factor 4α
LBD	Ligand-Binding Domain

LBP	Ligand-Binding Pocket
NCOA6	Nuclear Receptor Coactivator 6
NCoR	Nuclear Receptor Corepressor
NF1	Nuclear Factor 1
NLS	Nuclear Localization Signal
nt	Nucleotide
$OARE_{KI}$	Okadaic Acid Response Element
P450	Cytochrome P450
PAS	PER-ARNT-Sim
PB	Phenobarbital
PBREM	Phenobarbital-Responsive Enhancer Module
PCN	Pregnenolone-16α-carbonitrile
PDE4A5	Phosphodiesterase 4A5
PGC1α	Peroxisome-Proliferator-Activated Receptor Gamma Coactivator 1-Alpha
PIMT	PRIP-Interacting Protein with Interacting Methyltransferase Domain
PKA	Protein Kinase A
PP2A	Protein Phosphatase 2A
PRIP	Peroxisome-Proliferator-Activated Receptor (PPAR)-Interacting Protein
PXR	Pregnane X Receptor
PXRE	PXR-Response Element
RIF	Rifampicin
ROR	Retinoic-Acid-Receptor-Related Orphan Receptor
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RXR	Retinoid X Receptor
SMRT	Silencing Mediator of Retinoid and Thyroid Hormone Receptor
SNP	Single Nucleotide Polymorphism
Sp1	Specificity Protein 1
SRC	Steroid Receptor Coactivator
STAT1	Signal Transducers and Activators of Transcription 1
SV	Splice Variant
TAD	Transcription Activation Domain
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
TCPOBOP	1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene
TEF	Thyrotroph Embryonic Factor
TR	Thyroid Receptor
USF-1	Upstream Stimulatory Factor 1
VDR	Vitamin D Receptor
XAP2	X-Associated Protein 2
XRE	Xenobiotic Response Element
XREM	Xenobiotic Responsive Enhancer Module

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