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PHENOBARBITAL RESPONSE ELEMENTS OF CYTOCHROME P450 GENES AND NUCLEAR RECEPTORS[1]

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■ **Abstract** Phenobarbital (PB) response elements are composed of various nuclear receptor (NR)-binding sites. A 51-bp distal element PB-responsive enhancer module (PBREM) conserved in the PB-inducible *CYP2B* genes contains two NR-binding direct repeat (DR)-4 motifs. Responding to PB exposure in liver, the NR constitutive active receptor (CAR) translocates to the nucleus, forms a dimer with the retinoid X receptor (RXR), and activates PBREM via binding to DR-4 motifs. For *CYP3A* genes, a common NR site [DR-3 or everted repeat (ER)-6] is present in proximal promoter regions. In addition, the distal element called the xenobiotic responsive module (XREM) is found in human *CYP3A4* genes, which contain both DR-3 and ER-6 motifs. Pregnane X receptor (PXR) could bind to all of these sites and, upon PB induction, a PXR:RXR heterodimer could transactivate XREM. These response elements and NRs are functionally versatile, and capable of responding to distinct but overlapping groups of xenochemicals.

INTRODUCTION

In the 1960s, there was only one cytochrome P450 recognized in liver microsomes, and there were two distinct chemicals that induced cytochrome P450: phenobarbital (PB) and 3-methylcholanthrene. Since that time, this induction phenomenon has long been a major driving force to attract many scientists into cytochrome P450 research. It was soon realized that these chemicals induce different forms of cytochrome P450; then molecular biology enabled the recent explosive increase of the number of different cytochrome P450s to >500 (1). This was also the beginning of intensive efforts that have now resulted in the identification of xenochemical

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response elements and transcription factors that regulate these elements and in unveiling induction mechanisms at the molecular level (2–5). This review focuses on recent progress in understanding PB-inducible transcription of cytochrome P450 genes. For some aspects that are not covered in this review, readers should refer to recent articles (6–8).

Cytochrome P450s play the central role in xenochemical metabolism, as members of the heme-thiolate monooxygenase gene superfamily. By increasing the capability for metabolic detoxification and elimination, induction of cytochrome P450 is an integral part of the defense mechanism against xenochemical insult. It was already noted in the early 1950s that 3-methylcholanthrene fed to rats reduced the hepatocarcinogenic activity of certain aminoazo dyes by increasing their *N*-demethylation (9, 10) and that chronic administration of barbiturates gradually shortened PB-elicited sleeping time by inducing the metabolism of PB. Although induction is advantageous in most instances, inevitably it is also associated with pharmacological and/or toxicological consequences such as alterations in drug efficacy, drug-drug interactions (11), and metabolic activation of procarcinogens (12). Thus, induction of cytochrome P450 can be viewed as "an environmental friend and foe." Studies of the induction mechanism may provide insights into understanding general principles of maintaining biological homeostasis against environmental insults and also a way of controlling cytochrome P450 to be more friendly and beneficial to human health.

PB is the prototype of a large group of structurally unrelated chemicals that induce a large subset of cytochrome P450 genes: *CYP2A, CYP2B, CYP2C, CYP2H, CYP3A, CYP6A*, and *CYP102/106*. In addition to cytochrome P450s, PB concertedly induces a large number of other enzymes such as NADPH-cytochrome P450 reductase and specific transferases, increasing metabolic capability as a whole (13–15). The induction is largely limited to the liver, although other organs such as the brain can be targets for this induction. Because liver-derived cell lines do not respond to PB in induction of cytochrome P450 genes, the development of suitable primary hepatocyte cultures has led to recent progress in discovering regulatory mechanisms of PB induction. CYP2B has been the main object of studies of PB induction because PB most effectively induces this gene in the liver. With this gene, we begin a journey to search the PB response element (Figure 1).

CYP2B GENES

Search for Phenobarbital Response Element

The first cDNA and gene of CYP2B were cloned and sequenced in the 1980s (16, 17). Immediately thereafter, it was demonstrated that PB activates transcription of *CYP2B* genes (18, 19). However, the real progress in identifying a PB response element did not occur until the mid-1990s. First, expression of a chloramphenicol acetyltransferase gene-reporter construct driven by various 5′-flanking sequences in transgenic mice suggested the presence of PB responsiveness in a far (more than −800 bp) upstream region of the *CYP2B2* gene (20). The major

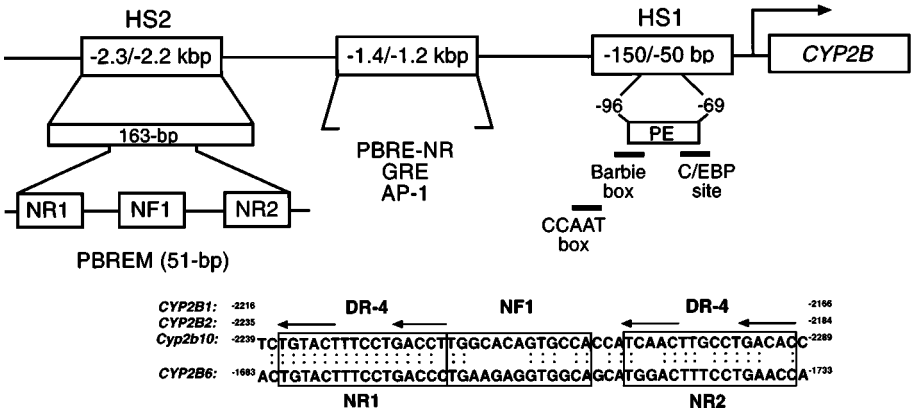

Figure 1 DNA elements found in the *CYP2B* genes.

break-through soon came from Anderson's laboratory, using rat primary hepatocytes, in which PB response activity was associated with a 163-bp DNA sequence at −2318 through −2155 bp of the *CYP2B2* gene (21). Subsequently, the PB response activity of this sequence was independently confirmed by using an in situ injection of the reporter gene constructs into rat liver (22). All of these results have agreed that the PB response element must reside within this 163-bp sequence of the *CYP2B2* gene designated a phenobarbital-responsive unit (23).

With mouse *Cyp2b10* and *Cyp2b9* genes, PB response activity of the 163-bp DNA was delineated to a 51-bp minimum sequence that could respond to PB. First of all, the corresponding DNA sequence at −2426 through −2250 in the PB-inducible *Cyp2b10* gene was identified and proved to be similarly activated by treatment with PB in mouse primary hepatocytes (24). DNase I footprinting on the DNA with mouse liver nuclear extracts defined six regions protected from digestion. Using this information, an additional deletion assay was conducted to delineate the PB response activity of the 163-bp DNA to the 69-bp sequence at −2365 through −2297. When base mutations within the corresponding 69-bp DNA of the noninducible *Cyp2b9* gene were introduced into the 69-bp response sequence of the *Cyp2b10* gene, this mutated response sequence completely lost its PB responsiveness. These findings provided genetic evidence that the PB response element is centered on the 69-bp DNA. Sequence analysis of the 69-bp DNA revealed the presence of two possible nuclear receptor (NR) motifs and a nuclear factor 1 (NF1) binding site. Keeping the presence of these motifs and the binding site in mind, further deletions and mutations were used to associate the PB response activity to a minimum sequence of 51-bp of DNA at −2339 through −2289 of the *Cyp2b10* gene, now called the phenobarbital-responsive enhancer module (PBREM) (25). The PBREM sequence was also found in the rat *CYP2B1*, *CYP2B2*, and human *CYP2B6* genes (26). Evolutionary conservation of PBREM in the *CYP2B* genes from mouse to human strongly supports the hypothesis that PBREM is a general PB response element.

Phenobarbital-Responsive Enhancer Module

The PBREM is characterized as a composite element consisting of two nuclear receptor (NR)-binding sites (NR1 and NR2) and an NF1-binding site (25). Both NR1 and NR2 are a DR-4 motif; a DR of imperfect half sites separated by 4 bases. Only the NR sites are essential for the PB response activity, although the NF1 site may be required to confer full PBREM activity. Mutation of either NR1 or NR2 decreased PBREM activity to one third of the wild-type activity in transfected primary hepatocytes, while simultaneous mutations of both NR sites abolished PBREM activity. PBREM sequences containing NF1 mutations retained significant residual activity in the PBREM. The nonessential role of an NF1 site was also clearly demonstrated in transgenic mice bearing a 5′-flanking region of the *CYP2B2* gene with a mutated NF1 site (27). This mutated DNA responded to PB as efficiently as did the wild-type DNA in the mice. In liver in vivo, however, a large alteration of chromatin structure occurred in the PBREM region after PB treatment, and the NF1 site appeared to be a core region of this alteration (28). The role of the NF1 site in modulating the PBREM-phenobarbital-responsive-unit activity should not be ruled out at the chromatin level.

PBREM is capable of responding to various PB-type inducers in addition to PB (25, 26, 29). These inducers include clotrimazole, chlorpromazine(CPZ), metyrapone, acetone, methyl isobutyl ketone, isoamyl alcohol, pyridine, 2,2′,4,4′-tetrachlorobiphenyl, 2,2′,5,5′-tetrachlorobiphenyl, 2,3,3′,4′,5,6-hexachlorobiphenyl, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), dieldrin, 1,1,1-trichloro-1,2-bis(*o*,*p*′-chlorophenyl)ethane (*o*,*p*′-DDT), methoxychlor, and camphor. On the other hand, neither 3-methylcholanthrene (CYP1A1 inducer), dexamethasone (CYP3A inducer), clofibrate (CYP4A inducer), nor 1,4-bis[2-(3-chloropyridyloxy)]benzene (an inactive derivative of TCPOBOP) activated PBREM. Thus, PBREM has emerged as a versatile response element that can specifically respond to various PB-type inducers, leading to the induction of *CYP2B* genes in the mouse, rat, and human. To begin to answer to the question of how such diverse chemicals activate the same response element, a transcription factor that regulates PBREM must first be identified.

Nuclear Constitutive Active Receptor

Sequence comparison of PBREMs revealed that the NR1 site (RGGTCAggaaAGTACA) is the most conserved element; mouse and rat NR1 sites are identical, and they differ only by 1 base from their human counterpart, suggesting that the NR1 site may play the key role in regulating PBREM in response to PB. Since the NR1 site is most conserved within the PBREM, efforts were concentrated on finding a nuclear protein that binds to this site (30). Two different approaches were concurrently taken to identify a binding protein to NR1. The first approach was to search for known NRs by using a transient transfection assay. The second was to purify a nuclear protein by using NR1-affinity chromatography. To accomplish this, we first cotransfected PBREM with various expression vectors of NRs, such as retinoid

X receptor (RXR), constitutive active receptor (CAR), liver X receptor, thyroid receptor-α, hepatocyte nuclear factor 4 (HNF4), and chicken ovalbumin upstream promoter-transcription factor (COUP-TF) in HepG2 and HEK293 cells. Among these liver-enriched NRs tested, only CAR was able to activate PBREM in the cotransfected cells. The nuclear CAR was originally characterized as a constitutive activator of an empirical set of retinoic-acid response elements, meaning that CAR activated the response element in the absence of retinoic acid (31). Consistent with the fact that CAR activated the response element by forming a heterodimer with RXR, coexpression of RXR resulted in a synergistic increase of PBREM activity in the CAR-transfected cells. As expected from these findings, a gel shift assay confirmed that an in vitro translated CAR alone did not bind to NR1, but its mixture with the similarly prepared RXR bound to NR1 specifically. Thus, it appeared that a CAR:RXR heterodimer activated the PBREM.

To purify a nuclear protein that binds to NR1, two different DNA resins were used for affinity chromatography: NR1-(AGGTCAGGAAAGTACA) and NR1′-conjugated resins (30). NR1′ [AGTTCAGAAAAGTACT (underlined bases differ from NR1] is the mutated NR1 found in the noninducible *Cyp2b9* gene. Western blot analysis of the affinity-purified fractions of mouse liver nuclear extracts showed that the NRs CAR and RXR were enriched on NR1 but not NR1′ resins and only from the PB-induced nuclear extracts. Moreover, protein microsequencing of the stained bands confirmed that CAR and RXR were included in the purified fraction from NR1 resin of PB-induced nuclear extracts. By forming a heterodimer with RXR, CAR appeared to be a nuclear factor capable of binding to NR1, and the binding occurred after treatment with PB. Transfection assays using the NR1-tk-reporter plasmid revealed that NR1 alone was activated by CAR, indicating that the binding of CAR:RXR to NR1 could be sufficient to activate PBREM. Because a mixture of the in vitro-translated CAR and RXR also exhibited a weaker binding to NR2, CAR:RXR may bind concurrently to both NR1 and NR2 during the activation of PBREM in the transfected cells. The cotransfection of mCAR resulted in expression of the endogenous *CYP2B* gene and the activation of PBREM in HepG2 cells (26). Moreover, a CAR-null mouse has been produced by using gene targeting, in which the induction of the *Cyp2b10* gene by PB or TCPOBOP was impaired (32). These results have provided the ultimate evidence that CAR is the receptor that can regulate PB induction of the *CYP2B* gene. A major enigma with this activation mechanism, however, was that CAR activated PBREM in the absence of PB or PB-type inducers in HepG2 cells. If CAR is to be a transcription factor that activates PBREM in response to PB, the receptor function must be repressed in unexposed liver in vivo. We discuss this in a later section of this article.

DNA Elements Other Than the Phenobarbital-Responsive Enhancer Module

Before the in vitro and in vivo systems (e.g. primary hepatocyte, transgenic mouse, and direct injection of DNA) were readily available for assaying PB-responsive

activity of DNA functionally, a search for a PB response element relied on nuclear protein-binding assays and was largely limited to the proximal promoter regions of *CYP2B* genes. Although various sequences have been defined as nuclear protein-binding sites in the promoter regions, a peer consensus has not yet emerged with respect to functional roles of these sequences and elements in PB induction. We have only briefly described DNA elements in the proximal promoter regions of *CYP2B* genes, because these have already been summarized in recent reviews (13, 33). Two DNase I-hypersensitive sites appeared on the *CYP2B2* gene after PB induction; one was in a proximal promoter (HS1), and the other one was at a distal −2.2-kbp region (HS2) (34). Although the HS2 region includes PBREM, the HS1 region contains the previously reported sequences and elements, such as a Barbie box (see below), CCAAT box, CCAAT-enhancer binding protein (C/EBP)-binding site, and other nuclear protein-binding sites. Some findings with these proximal sequences are quite consistent. Deletion or mutation of a putative C/EBP-binding site (at around −65 through −45 bp) dramatically decreased promoter activity of *CYP2B* genes in transient transfection assays (25). Although this site was neither responsive to PB nor did its mutation affect PB-enhanced reporter gene activity, it may regulate a basal transcription activity of the genes. No specific protein binding was observed to the Barbie box and deletion or mutation of the Barbie box did not affect PB responsiveness of *CYP2B* genes. A 27-bp DNA sequence [called a positive element (PE) and located at −96 through −69 bp] overlapping with the Barbie box and C/EBP site was repeatedly shown to have PB-responsive activity by using in vitro transcription and in vivo gene delivery systems (35–37).

Other possible regulatory elements were also located in the −1.4- through −1.2-kbp region of *CYP2B* genes: a functional glucocorticoid response element (GRE) at position −1357 bp (38) and an Activator protein-1 site at −1441 bp (39). A DNA sequence (−1404 through −971) of the *Cyp2b10* gene was associated with a minor PB response in mouse primary hepatocytes, in which there is an NR-binding motif (PBRE-NR) similar to NR1 within PBREM (40). Yet another 1.3-kbp 5′-flanking sequence of the *CYP2B1*gene also exhibited a PB response in transgenic mice (41). Thus, the elements within the −1.4- through −1.2-kbp region may account for some degree of total PB responsiveness of *CYP2B* genes.

CYP3A GENES

Dexamethasone/Rifampicin Response Element/Xenobiotic-Responsive Module

The *CYP3A* genes were originally characterized by their nonclassical induction by glucocorticoids (42, 43), and they are also known by their induction by macrolide antibiotics such as rifampicin (44). In addition, PB often induces these *CYP3A* genes. Response activity of various proximal promoter sequences to the typical CYP3A inducers (e.g. dexamethasone, pregnenolone16-carbonitrile and

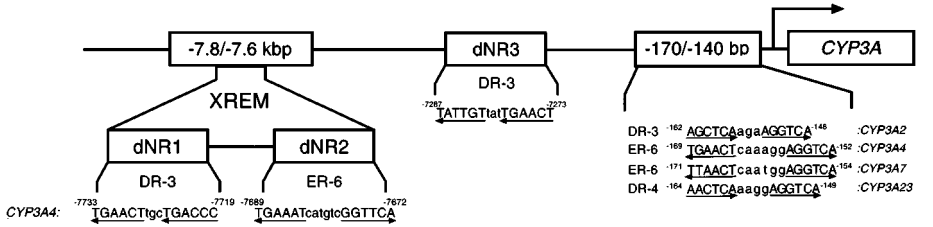

Figure 2 DNA elements found in the *CYP3A* genes.

rifampicin) was examined in primary hepatocytes or liver-derived H4IIE cells. As depicted in Figure 2, common enhancer elements emerged: 6β A-C in CYP3A2 (45), consensus II in CYP3A4 (46), and DexRE-1 in CYP3A23 (47, 48). These common elements are found in approximately -150-bp regions of *CYP3A* promoters, and they contain NR sites as DR-3, DR-4, or ER-6 motifs (49–51). Having the relatively low activation of these proximal elements by rifampicin, Goodwin et al took a courageous step to search for an additional response element up to the -13-kb region of the *CYP3A4* gene, and they identified a 230-bp distal element [called the xenobiotic-responsive enhancer module (XREM)] at -7836 through -7607 of the *CYP3A4* gene (52). A 417-bp proximal promoter sequence (-365 through $+52$) of the *CYP3A4* gene did not respond to either dexamethasone or rifampicin in HepG2 cells. Placing XREM in front of the 417-bp DNA conferred a strong response to both inducers. Moreover, PB also activated XREM in HepG2 cells. XREM contains two NR-binding sites—dNR1 (DR-3) and dNR2 (ER-6)—that are separated by 29 bases. Neither of these NR sites alone seems to regulate XREM; a mutation of dNR1 decreased response activity only 30–40%, whereas that of dNR2 increased it $\sim$20–30%. Even when both dNR1 and dNR2 were simultaneously mutated, XREM retained $>50\%$ of the original activity. Mutation of all dNR1, dNR2, and proximal consensus II/prPXRE (ER-6) sites reduced the activity to $<20\%$ of the wild-type XREM, suggesting that all three NR sites may be essential for XREM to acquire its PB response activity. An additional NR site (dNR3) was found in several hundred bases downstream from XREM, and the simultaneous mutation of dNR3 may abolish the XREM activity. Although XREM should expectedly be conserved in other inducible *CYP3A* genes, its presence has not yet been reported in these genes.

Nuclear Pregnane X Receptor

Database searching and subsequent cDNA cloning resulted in the discovery of a new mouse orphan NR designated pregnane X receptor (PXR) (49). Rat, rabbit, and human counterparts of mouse PXR have also been cloned (50, 53–56). Various chemicals that activate PXR turned out to be CYP3A inducers, thus identifying PXR as an NR that mediates induction of *CYP3A* genes (49). Gel-shift assays showed that, by forming a heterodimer with RXR, PXR could bind to all

dNR1, dNR2, and proximal pregnane X receptor response element sites, whereas a cotransfection assay confirmed that PXR:RXR activated XREM in response to dexamethasone, rifampicin, and other CYP3A inducers (52). PB also activated PXR and XREM in HepG2 cells. Taking the CAR-PBREM pathway into consideration, it now seems that PB could activate multiple NRs to induce different *CYP* genes via distinct response elements. Consequently, PB induction may be regulated through multiple pathways with different mechanisms and functional overlaps between these NRs, and elements will become more evident in future studies.

CYP2H GENES

These genes were isolated for PB-inducible cytochrome P450s in chicken liver (57). Like the *CYP2B* and *CYP3A* genes in mammalian liver, the *CYP2H* genes can be activated by PB in chicken liver (58, 59). Using primary hepatocytes prepared from chick embryos, PB-response activity was associated with two different DNA fragments in an upstream region of the *CYP2H1* gene: a 1351-bp DNA at −5900 through −4550 and a 557-bp DNA at −1956 through −1400 (60). Within the 556-bp DNA, the 240-bp fragment (−1640 through −1400) was found to retain full enhancer activity in response to PB. Most recently, an NR-binding site DR-4 motif (−1637 through −1622) has been identified within the 240-bp fragment (61), which is similar in its sequence (TGAACTTCCTTGCCCT) to the NR1 of the *CYP2B* genes. Various mutations within the DR-4 abrogated PB responsiveness of the 264-bp enhancer sequence at −1657 through −1393 (a considerable overlap with the 240-bp fragment) in a chicken hepatoma cell line. Thus, NR1 and its PB response activity appear to be evolutionarily conserved from avian to human *CYP* genes. Putative binding sites for various transcription factors such as NF1, AP1, HNF1, GRE, C/EBP, SREBP, and CHOP are also present in the 240-bp fragment or the 264-bp enhancer sequence.

CYP102/*CYP106* GENES

Studies by He & Fulco (62), Shaw & Fulco (63), Patel & Omer (64), and Liang et al (65) have resulted in the discovery of an elegant mechanism by which induction of the *CYP102* and *CYP106* genes is regulated by barbiturates in *Bacillus megaterium*. The underlying principle of this mechanism is the derepression of the *CYP* genes by barbiturate-mediated removal of a repressor (Bm3R1) from a 17-bp regulatory sequence, referred to as the Barbie box (62–65). Another *bm1p1* gene produces the Bm1P1 protein that functions as an activator by interfering with interaction of Bm3R1 and the Barbie box. This mechanism of PB induction has been seriously challenged in recent work by Shaw et al (66). Neither genetic disruption of the *bm1p1* gene nor removal of the Barbie box sequence affected CYP106 induction

by PB. The *bm1p1* gene, however, may be involved in repression of the basal expression of the *CYP101* gene (67, 68). A characteristic AAAG motif of the Barbie box sequence can also be found in proximal promoter regions of many mammalian PB-inducible *CYP* genes, including rat *CYP2B1, CYP2B2*, and *CYP3A2* and rabbit *CYP2C1* (64). However, the induction of the *CYP2B2* gene occurs even after removal of this sequence (22). In the *Cyp2b10* gene, a large insertion breaks the Barbie box in the middle of its consensus sequence (40). No Barbie box sequence is present within the known distal PB response elements, PBREM (in the *CYP2B* gene), XREM (in the *CYP3A* gene), and the 240-bp fragment (in the *CYP2H* gene).

CYP6 GENES

PB response activity was examined −1.3 kb upstream of the 5′-flanking region of the housefly Drosophila cytochrome P450 *Cyp6a2* gene (69). A transient-transfection assay using *Drosophila melanogaster* Schneider cells indicated that the PB response element resides within the −428 bp of the translation start site. The *CYP6D* gene in *Musca domestic* is also induced by PB, although neither *cis*-acting enhancer elements nor *trans*-acting factors have been reported (70).

OTHER *CYP* GENES

CYP2A1, CYP2A5, CYP2A14 (71), and various CYP2Cs have been reported to be induced by PB. However, PB response has not been associated with any DNA sequences of these *CYP* genes. Recently produced CAR- or PXR-null mice may be useful in discovering whether any of these receptors also regulate the induction of these *CYP* genes.

MECHANISM OF PHENOBARBITAL INDUCTION

The NRs CAR and PXR have emerged as the key factors mediating activation of PB response elements such as PBREM and XREM, leading to the induction of *CYP2B* and *CYP3A* genes. Molecular and cellular mechanisms for how these receptors regulate the elements in response to PB are receiving major interest in current investigations. NRs are generally activated by direct binding of ligand, called a ligand-dependent mechanism. However, the receptors can also be regulated without their direct binding to ligand. In this ligand-independent mechanism, phosphorylation and/or dephosphorylation of receptors and/or associated factors often dictate receptor functions. These ligand-dependent and -independent mechanisms are often referred as to direct and indirect regulations, respectively. Increasing numbers of NRs are so-called constitutive active receptors such as CAR, in which the ligand-independent mechanism may be more critical. Although

it is natural to assume that PB binds to CAR or PXR to trigger receptor activation, we may be urged to remain open to the possibility that these xenochemical response receptors may be regulated differently from the steroid receptors. Taking all existing experimental evidence into consideration, CAR may play the major role in PB induction, and the receptor apparently mediates the induction by proceeding through at least two distinct steps that are regulated differently—nuclear translocation and the receptor activation in liver nucleus (Figure 3). Expression of the fluorescent protein-tagged receptors has clearly shown that CAR is retained in the cytoplasm of unexposed liver, while PXR is localized in the nucleus. To induce *CYP2B* transcription, CAR must translocate to the nucleus of the liver in response to PB treatment.

Nuclear Translocation of CAR

CAR is retained in the cytoplasm of liver cells from untreated mice and accumulates in the nucleus only after treatment with PB (72). Evidently, there was no significant binding of CAR:RXR to NR1 in liver nuclear extracts from untreated mice, while there was a dramatic increase in this binding in liver extracts from PB-treated mice. Subsequently, Western blot analyses as well as immunohistochemical analyses confirmed that CAR was nearly absent in the liver nuclei of untreated mice, although it accumulated rapidly in the nucleus after PB treatment in a time-dependent manner before the increase of CYP2B10 mRNA. In addition to PB, treatments with various PB-type inducers such as CPZ, *o.p′*-DDT, and TCPOBOP also elicited the nuclear accumulation of CAR in mouse liver. Thus, CAR appeared to translocate to the nucleus in response to PB and other PB-type inducers, although nuclear export remains an alternative mechanism for the nuclear accumulation of the receptor. Nevertheless, the exclusion of CAR from the nucleus provides a mechanism that prevents the activation of *CYP* genes in the absence of inducers.

Our knowledge of the mechanism by which PB elicits the nuclear translocation is limited at the present time. Upon binding to an agonist, some steroid receptors are dissociated from their cytoplasmic complex, such as heat shock proteins, immunophilins, and p23 proteins, and these receptors translocate to the nucleus (73–75). Is the translocation of CAR also initiated by receptor binding to PB? The answer to this question may be “no,” but future research must prove or disprove this hypothesis. TCPOBOP induces *Cyp2b10* genes at 10 nM compared with other PB-type inducers that require micromolar to millimolar concentrations to induce the gene, thus being the best candidate for its binding to be demonstrated. In fact, it has recently been shown that TCPOBOP can directly bind to mCAR (76, 77), but not to hCAR in an in vitro binding assay. In these assays, PB did not bind to either mCAR or hCAR. Although hCAR did not bind to TCPOBOP, the receptor was capable of translocating to the nucleus in mouse liver in vivo after treatment with TCPOBOP. The direct binding, therefore, appeared not to be essential for nuclear translocation, at least for hCAR.

Then what else can regulate the translocation of CAR in response to PB? A clue toward understanding the translocation came from the known fact that okadaic acid (OA, a specific inhibitor for protein phosphatase 2A) represses the *CYP2B* induction by PB in rat and mouse primary hepatocytes (78, 79). As it turned out, OA pretreatment resulted in the repression of the nuclear translocation of CAR in the PB-induced primary hepatocytes (72). In transfected HepG2 cells, on the other hand, CAR spontaneously localized to the nucleus, and OA treatment did not affect either the nuclear localization of CAR or PBREM activity. This means that OA repression of the PB-induced nuclear localization does not seem to be regulated by the simple binding of OA to the receptor. PB may activate a type of protein phosphatase, and the phosphatase activity may be involved in the nuclear translocation of CAR in liver in vivo. Although the OA-sensitive nuclear translocation of CAR occurs as an initial step of PB induction, CAR appears to undergo a subsequent activation process in the nucleus.

Nuclear Activation of CAR

Immediately after being accumulated in the nucleus, CAR does not seem to be capable of activating PBREM and inducing the *Cyp2b10* gene in mouse primary hepatocytes (80). When mouse primary hepatocytes were pretreated with the Ca^{2+}/calmodulin-dependent kinase (CaMK) inhibitors KN-62 and KN-93, these hepatocytes did not respond to PB by either activation or induction. Similarly, both KN-62 and KN-93 also inhibited the mCAR-mediated activation of PBREM in transfected HepG2 cells. Despite all of this, CAR was found to accumulate in the nucleus of the pretreated hepatocytes, indicating that the accumulated CAR was an inactive form of the receptor. How does PB activate the CAR in the nucleus?

Activation and/or repression of NRs is generally regulated by coregulators—coactivators and corepressors (81, 82). An NR protein consists of a DNA-binding domain, ligand-binding domain, and activation function domains (AF1 and AF2). Coregulators modulate NR activity in ligand-dependent and/or -independent mechanisms through their association and/or dissociation to either N-terminal AF1 or C-terminal AF2 domains or a corepressor-binding site encompassing the hinge region. CAR protein also contains these characteristic domains, except the AF1 domain. Although CAR is inherently active in the absence of ligands (i.e. inducers) in HepG2 cells, the AF2 domain is essential for receptor activity (83). It is, therefore, reasonable to speculate that the activation by PB of CAR in the nucleus may be regulated through coregulators. Roles of the coactivator steroid receptor cofactor-1 (SRC-1) in activating CAR have been reported, although reports are not always consistent. Forman et al showed the constitutive association of SRC-1 to mCAR, and the dissociation by androstenol of the CAR:SRC-1 complex (84). Consistent with the SRC-1 dissociation mechanism, androstenol also inhibited the constitutive expression of the endogenous *CYP2B6* gene and the activity of PBREM in HepG2 cells expressing mCAR (26). Moreover, treatment with 5 mM PB or 250 nM TCPOBOP reactivated the androstenol-repressed gene as well as

PBREM in these cells, although the concentrations were 5- to 10-fold higher compared with those that were effective in inducing the gene and activating PBREM in mouse primary hepatocytes. Contrary to the dissociation mechanism, Moore and his coworkers did not observe constitutive association of mCAR with SRC-1, although the association occurred when TCPOBOP was present in the assay (77). This finding is consistent with the hypothesis that the direct binding of TCPOBOP recruits SRC-1 to associate with mCAR, resulting in receptor activation. Thus, the regulatory role of SRC-1 is still enigmatic and finding the true coregulators that respond to PB induction remains of major interest.

An alternative to these ligand-binding mechanisms is an indirect regulation of CAR interaction with a coregulator through a signal transduction pathway. CaMK could be such a factor that activates CAR in the nucleus after PB exposure, because PB induction of the *Cyp2b10* gene is known to be inhibited by KN-62 and also by the intracellular Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate-acetoxymethyl ester (85). Consistent with this hypothesis, overexpression of CaMKII or CaMKIV resulted in enhancement of the CAR-dependent NR1 activity in HepG2 cells, and the CaMK inhibitors KN-62 and KN-93 repressed the PBREM activity and CYP2B10 induction in mouse primary hepatocytes (80). Neither KN-62 nor KN-93 inhibited the nuclear translocation of CAR in the primary hepatocytes, indicating that the PB activation of CAR is distinctly regulated in vivo in liver. In sharp contrast, OA repressed the nuclear translocation, but it did not inhibit activation. Multiple phosphorylation/dephosphorylation signal pathways could be involved in regulating the translocation and/or activation, placing the multiple shields preventing CAR from activating genes in the absence of exposure to stimuli (Figure 3). Further investigation is needed to identify the specific phosphatases, kinases, and proteins modulated by these enzymes. Once the kinases and phosphatases and the coregulators modulated by these enzymes have been identified, we should be able to elucidate the activation mechanism of CAR by PB and the other PB-type inducers.

Activation of Pregnane X Receptor

Activation of PXR is primarily consistent with a ligand-dependent mechanism, meaning that the direct binding to PXR inducers activates the receptor. PXR is a constitutive inactive receptor and always resides in the nucleus regardless of the presence or absence of its inducers. Thus, the activation mechanism of PXR appears to be less complicated compared with that of CAR. While steroid hormone receptors such as estrogen receptor-α bind to their agonists at nanomolar concentrations, activation of most so-called nuclear orphan receptors including PXR and CAR require micromolar concentrations of inducers. This high concentration or low affinity makes it difficult to assay binding accurately by conventional methods. With a recently developed scintillation proximity binding assay in vitro, the relative binding activities of various CYP3A inducers have been determined as the degree of inhibition of ^{3}H-labeled SR12813 binding to PXR (86). PB exhibited

50% inhibition at 1 mM, compared with ∼60%, 30%, and 20% inhibition by 10 μM rifampicin, pregnenolone16α-carbonitrile, and dexamethasone, respectively. The most potent PXR activator, 5β-pregnane-3, 20-dione, inhibited the ^{3}H-labeled SR12813 binding nearly completely at 10 μM. In general, the inhibition rates were roughly correlated with their potency in activating response elements such as XREM in transfected CV-1 or HepG2 cells (52, 86). Thus, the direct binding of inducers appeared to be a rate-limiting step in PXR activation via recruiting the coactivator SRC-1 (76, 86).

Species differences in the function of PXR provide additional evidence supporting the hypothesis that PXR activation is dictated by direct binding of inducer to the receptor. CYP3A induction in different species depends on the types of inducers; for example, rifampicin is a good inducer only in rabbits and humans, pregnenolone16α-carbonitrile, only in rodents, and 5β-pregnane-3, 20-dione only in mice and humans (7, 87). This species-specific induction is reflected by the activation patterns of these chemicals on mouse, rat, rabbit, and human PXR in transfected cells, providing further evidence that PXR is regulated by its direct binding to the inducers.

FUTURE ISSUES

NRs have been found to play the major role in regulating the transcription of *CYP* genes (6–8). Among these receptors, recent receptor-null mice have confirmed that CAR and PXR regulate the xenochemical induction of the *CYP2B* and *CYP3A* genes, respectively. These *CYP* genes contain the enhancer elements that can be activated by the corresponding receptors after xenochemical exposures. Despite these recent developments in defining the response elements and identifying the NRs, apparent complication and diversity may keep us interested in PB induction for many years to come. Hereafter, we primarily focus on CAR and discuss future research directions, because CAR may play a major role in PB induction of cytochrome P450 genes.

Direct vs Indirect Mechanism

The question of whether PB regulates CAR by direct binding is still of key interest. Even with a sensitive in vitro binding assay, PB has not been shown to bind to CAR. Although this does not necessarily mean that PB does not bind to the receptor, it is leading us to widely investigate various mechanisms that might regulate CAR. CAR is retained in the cytoplasm in unexposed liver, and the nuclear translocation is the first step occurring in response to PB exposure. Identification and characterization of the cytoplasmic complex of CAR may provide a clue to understanding how PB translocates CAR into the nucleus. In addition to proteins that constitute cytoplasmic complexes of steroid receptors, some unique proteins such as protein phosphatase and kinase may also be included in the CAR complex

to regulate the OA-sensitive nuclear translocation of the receptor. Once the inactive nuclear CAR complex accumulating in nuclei treated with CaMK inhibitor such as KN-62 has been characterized, we may be able to investigate whether a CaMK directly activates the receptor. In response to PB exposure, cellular machinery such as importins and Ran may also operate to translocate CAR to the nucleus. In addition, the nuclear accumulation of CAR can be determined not only by nuclear translocation but also nuclear export or even by export alone. In defining these processes, we may be able to answer whether the direct binding of PB is essential for CAR for activating of gene transcription in liver. If CAR turns out not to be the target, the direct target of PB may then be identified.

The regulation mechanism of the hCAR is enigmatic at the present time. PB responsiveness of hCAR for nuclear translocation in mouse liver has been experimentally substantiated, whereas activation activity in transformed cells has not yet been demonstrated because no chemical has been found to repress the constitutive activity of hCAR. In contrast to mCAR, which can be repressed by xenochemicals such as androstenol, KN-62, and KN-93, the constitutive activity of hCAR appears not to be repressed by these chemicals. Moreover, TCPOBOP was shown to directly bind to mCAR but not to hCAR. Although all of these findings encourage us to consider that the receptors may be regulated through different mechanisms, the line of studies described in the previous paragraph may also help us to understand the real mechanism that regulates both receptors in response to PB exposure.

Genes That Can Be Regulated by CAR

PB treatment causes pleiotropic effects on liver function and coordinates the induction of xenochemical metabolizing capability. PB may induce >20 different genes in chicken primary hepatocytes (14). As many as 77 differentially displayed cDNAs have been detected in PB-induced mouse liver (88). Does CAR regulate PB-inducible transcription of the xenochemical-metabolizing enzymes other than *CYP2B* genes? Searches for cDNAs expressed to a higher level in the mCAR-expression g2car-3 cells over HepG2 cells have identified human bilirubin UDP-glucuronosyltransferase (*UGT1A1*) as one of the genes that can be upregulated by the CAR (89). Besides UGT1A1 and CYP2B6, other enzymes such as carboxyesterase and superoxide dismutase 3 were also overexpressed in g2car-3 cells (A Ueda & M Negishi, unpublished observation). Continuing this line of experiments using the additional techniques such as DNA microarray may provide us with a complete picture of the genes that can be regulated by CAR and the role of the receptor in its concerted ability to inducing the hepatic metabolic capability.

Receptor Cross-Talk

Considering that there is a significant overlap of CYP2B and CYP3A inducers, receptor cross-talk may occur frequently. When a xenochemical activates both CAR and PXR, one or both of these may induce *CYP* genes or other genes, depending

on a given condition. CAR activates the enhancer elements of the *CYP3A* genes: the proximal DR-3 and ER-6 motifs and the distal XREM in transfected cells (26, 76, 77). When XREM and clotrimazole are the receptor target and inducer, respectively, PXR is the primary receptor regulating the transactivation in CV-1 cells (76). However, this kind of the activation phenomenon may differ according to the types of inducers, receptors, enhancer sequences, and assay systems used. For instance, KN-62 repressed PB induction of *Cyp2b10* and *Cyp3a* genes in a similar manner in mouse primary hepatocytes (85, 90, 91), which is more consistent with a CAR-regulated mechanism for both *Cyp* genes. The recently developed receptor-null mice may help us to sort out the degree of cross-talk between the two receptors CAR and PXR, at least in mice.

Endocrine factors such as oxysterols; glucocorticoids; sex, thyroid, and growth hormones; and growth factors are not directly responsible for PB induction, but they are modulating factors in vivo (92–95). For instance, the PB induction of CYP2B was diminished about 30% in glucocorticoid receptor-null mice (96). These endocrine modulations may happen through NR cross-talk involving various NR motifs. PBREM contains NR motifs for estrogen receptor-related receptor (ERR), COUP-TF, and PXR (26, 97). The ERR-binding site overlaps with NR1 site and the ERR binding represses CAR-activated PBREM activity in HepG2 cells. HNF4 also showed binding to a region within PBREM (27). A putative glucocorticoid response element or a so-called accessory that is overlapped with the 5′- or 3′-regions of PBREM, respectively, is reported to modulate PB responsiveness of the *CYP2B2* gene (23). These motifs and sequences may serve as the sites at which various NRs and transcription factors cross talk. In addition, the modulation could be regulated through RXR, the heterodimer partner.

Pharmacological/Toxicological Implications

As a tumor promoter, treatment with either PB or TCPOBOP is known to produce hepatocarcinoma in rodents (98, 99), although similar tumor development has not yet been confirmed in humans. In general, PB and many PB-type inducers are often categorized as nongenotoxic carcinogens, and the mechanisms of nongenotoxic carcinogenesis have not been well understood. Peroxisome proliferator activated receptor α (PPARα)-null mice have shown a resistance to hepatocarcinogenesis caused by the receptor activator WY-14,643 (100). It will be interesting to investigate whether CAR and/or PXR may also play a key role in hepatocarcinogenesis by PB and PB-type inducers. Does CAR play roles in proliferation of the endoplasmic reticulum, changing glucose metabolism, increasing heme synthesis, and altering the redox potential in the liver? Once these questions have been answered, PB response elements (PBREM and XREM) and the receptors CAR and PXR can be used to develop a drug-screening system. Moreover, a gene switch system requiring a fine-tuning of gene expression can be constructed by using these elements and receptors. If these elements and receptors happen to be polymorphic in human populations, these polymorphisms could potentially be used for individual

diagnosis for susceptibility to therapeutic drugs and environmental toxicants and carcinogens.

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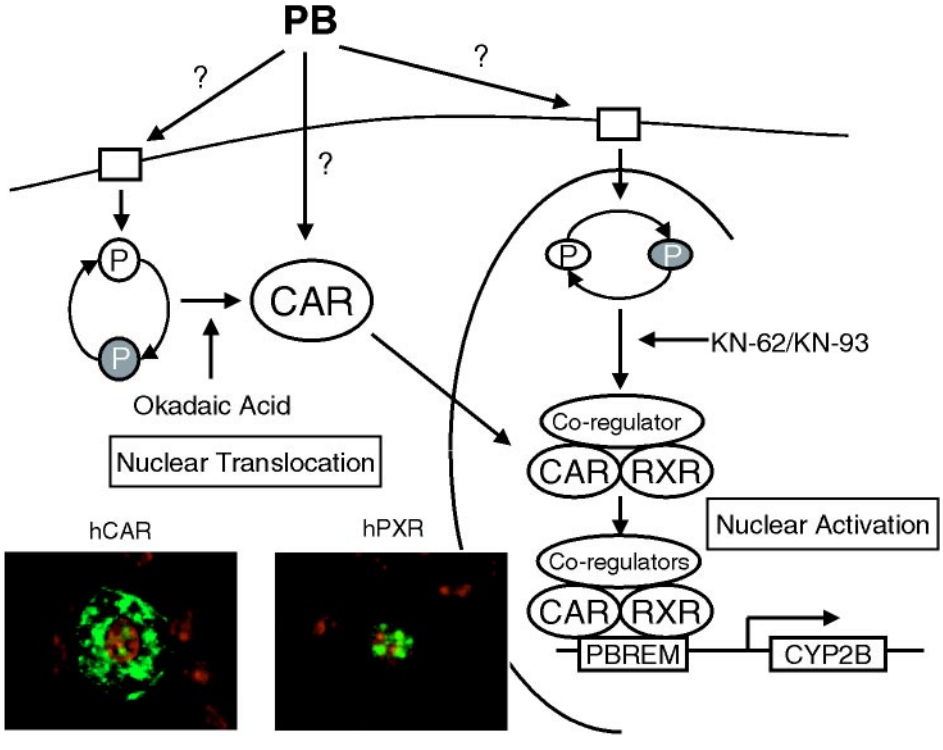

Figure 3 Working hypothesis of PB induction mechanism. The inset pictures show the cytoplasmic and nuclear localization of the green Øuorescent protein-tagged hCAR and hPXR, respectively, in the liver cells.