The Phenobarbital Response Enhancer Module in the Human Bilirubin UDP-Glucuronosyltransferase UGT1A1 Gene and Regulation by the Nuclear Receptor CAR

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The UDP-glucuronosyltransferase, UGT1A1, is the critical enzyme responsible for detoxification of the potentially neurotoxic bilirubin by conjugating it with glucuronic acid. For decades, phenobarbital (PB) treatment for hyperbilirubinemia has been known to increase expression of the UGT1A1 gene in liver. We have now delineated the PB response activity to a 290-bp distal enhancer sequence (~3483→3194) of the UGT1A1 gene. The enhancer contains 3 putative nuclear receptor motifs, and it was activated by the nuclear orphan receptor, human constitutive active receptor (hCAR), in cotransfected HepG2 cells. Bacterially expressed hCAR, acting as a heterodimer with in vitro-translated retinoid X receptor (RXRα), only bound to 1 of the 3 NR motifs, named gtNR1 in a gel-shift assay. Consistently, mutations of the gtNR1 site significantly decreased the activation by hCAR of the 290-bp DNA in transfection assays. Moreover, the 290-bp DNA was effectively activated in mouse primary hepatocytes in response to PB, offering an excellent clinical test for the examination of the responsiveness of the UGT1A1 to PB in the human population, particularly individuals with hyperbilirubinemia. (HEPATOLOGY 2001;33:1232-1238.)

Bilirubin IXα, one of the most toxic natural breakdown products in the body, is commonly associated with jaundice in neonates and young children. At extraordinarily high serum levels, it can cause mild to lethal neurotoxicity. On a daily basis, large amounts of the chromophore are formed in the spleen from heme salvaged from senescent red blood cells, as well as a lesser amount from the turnover of cytochrome P450. In mammals, before bilirubin can be effectively excreted by the biliary system,1 it must be transported to and glucuronidated2,3 in the liver via a specific UDP-glucuronosyltransferase.

The requirements for glucuronidation of bilirubin for its excretion were first recognized from the fact that a severe unconjugated hyperbilirubinemia persists for the entire life span of Crigler-Najjar (CN) type I patients4 and the Gunn rat,5 both of whom totally lacking bilirubin UDP-glucuronosyltransferase activity in liver microsomes.1 Later, less severe forms of the disease, CN type II and Gilbert’s type, were found to be distinct from the CN type I when phenotypes with low levels of glucuronidating activity for the metabolite were found.6-9 Phenobarbital (PB) treatment was shown to dramatically reduce the hyperbilirubinemia in an infant with CN type II disease.10 The chain of these events has led to the practice of treating patients whose hyperbilirubinemia was caused by clinical entities other than CN diseases with PB. Bilirubin is conjugated by the specific UDP-glucuronosyltransferase.9 UGT1A1, the gene that codes for the human bilirubin isozyme, has been characterized in the UGT1 complex locus.11 However, the mechanism by which PB brings about the increase in bilirubin transferase activity remains unknown.

Recently, the conserved 51-bp PB-responsive enhancer module (PBREM) has been defined in PB-inducible CYP2B genes.12 Moreover, the nuclear receptor known as constitutive active receptor (CAR) is identified as the transcription factor that regulates PBREM in response to PB induction.13 However, this enhancer and the CAR regulation have not been identified for the human UGT1A1 gene. The human CYP2B6 gene, not expressed in HepG2 cells, becomes activated in the cells transfected with a CAR-expression plasmid.14 If the induction by PB of the UGT1A1 gene is regulated by a mechanism similar to that of the CYP2B6 gene, CAR-expressing HepG2 cells are an excellent system to screen for potential human genes that can be regulated by the receptor, such as UGT1A1. In this article, we characterize a CAR-mediated enhancer sequence of the UGT1A1 gene, and provide experimental consideration for the mechanism by which PB activates transcription of the gene in human liver.

MATERIALS AND METHODS
Screening Atlas Arrays. HepG2 and g2car-3 cells14 were cultured in minimal essential medium supplemented with 10% fetal bovine serum. Total RNA was prepared from these cells using TRIZOL reagent.
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TTCAAATTCCTGGGATAGTGGATT-3

TCATAAA-3

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CA) and was sequenced.
g2car-3 cells compared with their expression in HepG2 cells. Although the degrees of the CAR involvement in their expressions are not clear at the present time, all these genes can be used in future studies to determine the CAR-mediated trans-activation mechanisms, as well as the biological roles of CAR in vivo.

Because PB is known to induce the UGT1A1 gene in liver in vivo, here we chose this gene for further characterization to identify DNA enhancer elements that can be regulated by CAR. To confirm that CAR, in fact, could regulate the expression of the UGT1A1 gene in g2car-3 cells, the cells were pretreated with a repressor of mCAR, androstenol, before induction by the most potent PB-type inducer TCPOBOP. The UGT1A1 mRNA was decreased by treatment with androstenol and was increased in TCPOBOP-induced cells (Fig. 1B and 1C), which is reminiscent of induction of the CYP2B6 gene.

**Deletion Assays.** In an effort to locate the CAR-regulated enhancer element in the UGT1A1 gene, various DNA fragments generated from a 12-kbp 5′-flanking region of the gene were generated and placed in front of the reporter luciferase gene. These deletion constructs were examined for their enhancer activity in HepG2 cells cotransfected with hCAR-expression plasmid (Fig. 2). Only the U2K fragment (−5193/−3092)
displayed prominent enhancer activity, whereas the other DNA fragments were not activated by hCAR at all. Subsequently, further deletion assays were performed on the U2K fragment to delineate a minimal sequence that could be activated by hCAR (Fig. 3). A full enhancer activity was associated with the 462-bp 3′-fragment (−3553/−3092), while the 5′-fragment (−5193/−3554) exhibited no activity. Within the 462-bp fragment, a middle 140-bp fragment activated the reporter gene approximately 2-fold, suggesting that this fragment may be a core of the enhancer activity. In fact, including additional 5′- and 3′- bases to the 140-bp fragment, a 290-bp fragment (−3483/−3194) was fully capable of activating the reporter gene in the cotransfected HepG2 cells.

**Mutation Assays.** Nucleotide sequence analysis of the 290-bp DNA revealed 3 potential nuclear receptor motifs that are each separated by about 90 bases (Fig. 4). From the 5′-end, these motifs were named NR4 (DR-4 motif), gtNR1 (DR-4 motif), and NR3 (DR-3 motif), respectively. The sequence of gtNR1 is highly homologous to the NR1 site within the 51-bp PBREM conserved in the CYP2B genes. To define roles of each NR motif in hCAR-dependent activation of the 290-bp DNA, these motifs were mutated singularly or simultaneously. The mutated DNAs were constructed into luciferase reporter gene plasmids, and subjected to transient transfection assays in HepG2 cells cotransfected with the hCAR-expression plasmid (Fig. 5). Mutation of gtNR1 resulted in an 80% decrease of the CAR-dependent enhancer activity. On the other hand, single mutations of either NR4 or NR3 retained about 50% of the original activity. When all 3 motifs were simultaneously mutated, the 290-bp DNA completely lost its enhancer activity. These results indicate that gtNR1 plays the most significant role in the activation of 290-bp DNA by hCAR. Consistent with this, a mixture of GST-hCAR and *in vitro*-translated hRXRα bound to gtNR1, but not to NR3 and NR4, in gel-shift assays (Fig. 6). Thus, gtNR1 appeared to be the only binding site of the hCAR:hRXRα heterodimer. In
Thus, the 290-bp DNA appeared to be an effective PB response conferring a full enhancer activity to the 290-bp DNA (Fig. 5). Our finding that all 3 nuclear receptor motifs were required for repression by CAR, gtNR1 could only be activated by the receptor in the context of 290-bp gtPBREM, and NR4 and NR3 seem to be essential for gtNR1 to confer its full enhancer activity. NR1 may be a much stronger enhancer and can be activated by CAR, whereas gtNR1 might be a weaker enhancer that requires additional factors for its activation. However, these different strengths of gtNR1 and NR1 reflect a somewhat lesser degree of PB responsiveness of the UGT1A1 gene compared with CYP2B genes.

PB Responsiveness. The responsiveness of the 290-bp DNA to PB was examined with 2 different systems: HepG2 cells and mouse primary hepatocytes. To examine PB responsiveness of a given CAR-mediated trans-activation in transfected cells such as HepG2, constitutive activity of this receptor must be repressed by compounds such as androstenol before PB treatment. For this purpose, the 290-bp DNA-luciferase reporter plasmid was cotransfected with either the mCAR- or hCAR-expression vector into HepG2 cells (Fig. 7A). As expected, when mCAR was cotransfected, androstenol treatment repressed the enhancer activity of 290-bp DNA, and the repressed expression was reactivated by TCPOBOP. To a lesser degree, the same repression and reactivation were also observed with hCAR. Because hCAR was better than mCAR with respect to their constitutive activation of 290-bp DNA, and once an effective repressor of hCAR has been identified, the PB response capability of hCAR may be more clearly demonstrated in the future. Nevertheless, in response to PB, both mCAR and hCAR could activate the 290-bp DNA in PB.

To obtain more direct indication for the PB responsiveness of 290-bp DNA, the same reporter plasmid was transfected into mouse primary hepatocytes, and the hepatocytes were treated with PB or saline solution for 24 hours. Subsequently, cell lysates were prepared for luciferase assays (Fig. 7B). The treatment with PB resulted in over 5-fold induction of the 290-bp DNA-enhanced luciferase activity. This response appeared to be mediated by CAR, because the gtNR1-tk-luciferase reporter gene alone was also activated by PB treatment, although the gtNR1 responded to PB to a lesser extent compared with the 290-bp DNA or the NR1 of the Cyp2b10 gene. This weak response of gtNR1, however, was consistent with our finding that all 3 nuclear receptor motifs were required for conferring a full enhancer activity to the 290-bp DNA (Fig. 5). Thus, the 290-bp DNA appeared to be an effective PB response element in mouse primary hepatocytes, and this PB response activity was mediated by CAR. We designated the 290-bp DNA the PB-responsive enhancer module of UGT1A1, or gtPBREM.

General Discussion. The gtPBREM is a composite element consisting of 3 NR motifs and probably still-unknown cis-acting sites. Like the 51-bp PBREM of CYP2B genes, gtPBREM can be regulated by the nuclear receptor, CAR, in response to PB induction. However, when gtPBREM was compared with the 51-bp PBREM of the CYP2B genes, major structural and functional differences became evident. In the PBREM, 2 NR sites (NR1 and NR2) reside close to each other, separated only by 18 bases that constitute the nuclear factor 1 binding site. There is no apparent nuclear factor 1 binding site in the gtPBREM. The most striking difference of gtPBREM is the functional role of gtNR1, the only CAR binding site. In sharp contrast to NR1, which is a sufficient1 element activated by CAR, gtNR1 could only be activated by the receptor in the context of 290-bp gtPBREM, and NR4 and NR3 seem to be essential for gtNR1 to confer its full enhancer activity. NR1 may be a much stronger enhancer and can be activated by CAR, whereas gtNR1 might be a weaker enhancer that requires additional factors for its activation. However, these different strengths of gtNR1 and NR1 reflect a somewhat lesser degree of PB responsiveness of the UGT1A1 gene compared with CYP2B genes.

PB, with its ability to induce the UGT1A1 gene, can be an effective therapeutic drug to treat CN type II patients in whom expression of bilirubin transferase activity is low. These patients benefit from PB treatment by the increased expression of the partially active bilirubin transferase enzyme.18 Polymorphisms have been found within the proximal region of the UGT1A1 gene, and have been associated with Gilbert’s syndrome.19 While the TATA box of the UGT1A1 gene consists of the (A(TA)) nTAA sequence, the TA dinucleotide is repeated 6 times in the presumed wild-type gene and 5, 7, or 8 times in “mutant” alleles.10-21 The (A(TA)) nTAA exhibited approximately 4-fold higher transcription activity than the A(AT) TAA in transient transfection assays. The A(TA) TAA is not a PB response element, because a proximal promoter including this sequence was not capable of activating the transcription in
response to PB. The distal PB response element, gtPBREM, might be able to enhance transcription activity of the weak A(TA)$_7$TAA, resulting in induction of the UGT1A1 enzyme to decrease unconjugated bilirubin. In addition, mutation of gtPBREM could become a cause of hyperbiliruminemia, if the enhancer module is regulated by some endogenous factors. Such mutations might alter patient susceptibility to responding to PB treatment, as seen in some CN-type II patients. The effects could be more critical for patients with an additional mutation in the coding region that produces an inefficient UGT1A1 enzyme. Finding gtPBREM polymorphisms may provide an insight into understanding the diseases, as well as developing better therapeutic methods. If such polymorphisms are identified, they would also aid in the study of the molecular mechanism that regulates gtPBREM and the UGT1A1 gene in vivo.

REFERENCES


