Characterization of a Phenobarbital-responsive Enhancer Module in Mouse P450 Cyp2b10 Gene

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Induction of drug- and carcinogen-metabolizing cytochrome P450s by xenobiotic chemicals is a common cellular defense mechanism, usually leading to increased detoxification of xenobiotics but sometimes, paradoxically, to formation of more toxic and carcinogenic metabolites. Phenobarbital (PB) is an archetypal representative for chemicals including industrial solvents, pesticides, plant products, and clinically used drugs that induce several genes within CYP subfamilies 2B, 2A, 2C, and 3A in rodents and humans. Although the transcription of these CYP genes is activated by PB, the associated molecular mechanisms have not yet been elucidated. Here we have analyzed, in detail, enhancer activity of a far upstream region of mouse Cyp2b10 gene and report a 132-base pair PB-responsive enhancer module (PBREM) with a 33-base pair core element containing binding sites for nuclear factor I- and nuclear receptor-like factors. Mutations of these binding sites abolish the ability of PBREM to respond to inducers in mouse primary hepatocytes.

The cytochrome P450s (CYPs) comprise a superfamily of heme-thiolate proteins with diverse functions from the synthesis and degradation of steroid hormones and fatty acid derivatives to metabolism of xenobiotic chemicals such as drugs, industrial chemicals, environmental pollutants, and carcinogens. Induction of P450s by xenobiotic chemicals is a common phenomenon, conserved throughout the vertebrate kingdom, insects, and bacteria. Xenobiotic inducers can be sorted into distinct classes based on the subsets of P450s induced; for instance, polycyclic aromatic hydrocarbons and peroxisome proliferators are known to activate CYP1A and CYP4A genes through ligand-dependent aryl hydrocarbon and peroxisome proliferator-activated receptors, respectively. Phenobarbital (PB) represents a large number of structurally unrelated chemicals that induce the same set of CYP genes including members within subfamilies 2A, 2B, 2C, and 3A, with CYP2B forms being activated most effectively (for review, see Ref. 10). PB also induces various transferases and enzymes of heme metabolism and affects processes of cell growth and cell-cell communication (10). The signaling pathways through which PB acts to regulate the induction process of CYP genes are mostly unknown due to the loss of PB response in hepatoma cells and the lack of other reliable in vitro assays. Also, the findings on PB-responsive DNA regulatory elements of these CYP genes have been quite inconsistent (11–16). We have addressed the above problems by developing a primary hepatocyte culture in which both endogenous and transfected Cyp2b10 genes remain transcriptionally inducible by PB-like chemicals (17). Using this culture system, we now identify and dissect a PB-responsive enhancer module PBREM from the mouse Cyp2b10 gene and show that binding sites for both nuclear factor I- and nuclear receptor-like factors are involved in induction.

**EXPERIMENTAL PROCEDURES**

Plasmids—The 177-bp Cyp2b10 DNA fragment (−2426 to −2250 bp) from plasmid BglPst4 (17) was amplified using primers 2B10-S (5′-caggagattgtcctggatcaggacagca) and 2B10-A5 (5′-caggagattcagtgcagatcacaacca). Cyp2b9 gene DNA fragment (−898 to −765 bp) (18) was amplified from BamHI-digested CD-1 mouse genomic DNA (2 μg) with primers 2B9-S (5′-ettgattacaatcttcgtggatcaaggaggaggaaagga) and 2B9-A5 (5′-ettgattacacgctttgcagagacaaaaactcgggtgtgctatcatt). The amplified DNAs were digested with BamHI and ligated into BamHI site of pBLCAT2 (tkCAT) plasmid (19). Various deletions of Cyp2b10 177-bp fragment (depicted in Fig. 3) were done using appropriate 20–24-mer primers harboring a BamHI site at 5′-ends for cloning as above. Internal deletions of elements pB’ and pC were generated using primers 2B10-S, 2B10-A5, and 20-mer primers with a 5′-end EcoRI site annealing to elements pB, pB’, pC, and pD. In wild-type Cyp2b10 fragment, this resulted in a 3-bp mutagenesis of the minimal enhancer construct pB-C (−2336 GTACTT to GaAtTc) without any change in the spacing between elements pB’ and pC. All mutations from the wild-type sequence shown hereafter are indicated by underlined, lowercase characters. To expedite the mutation of pC, the spacer region between pC and pD was first changed to an XbaI site (−2298 GCTGTA to −2280 GATGTA), and pC was then mutated by changing six nucleotides from Cyp2b10 sequence to those in corresponding Cyp2b9 position by primer MUT-pC (−799 tgtctgtagAAGTCATGTCGACACTGTTGACAAAGATTAGCAAAACCATGAGAAGAAAGA). Mutagenesis of the minimal enhancer construct pB-pC (−3236−2342) were done using primers B’-mut1 (−3234 cttgattaccAACATGCGGTGACAGACACGAGACACCA) and B’-mut2 (−2344 cttgattacACCATGCGGTGACAGACACGAGACACCA). pC-NF1 (−2297 cttgattacGCAGTGTGATGATGACTGATGATGACTGATGACTGATGACTGAC) and pC-NRMs (−2297 cttgattacGCCAGTTGAGTGACGCACCAGACACGAAAGATAG) plasmids (−4300CAT, −1404CAT, and −566CAT were described previously (17). Plasmid −2937CAT was constructed by amplifying the Cyp2b10 region between −2397 and −1404 bp using proof-reading Pfu DNA polymerase and primers containing HindIII sites. The amplified DNA was inserted into HindIII-digested −4300CAT plasmid. Plasmid −1850CAT was generated from −4300CAT by partial PvuII digestion and self-ligation. Appropriate recombinant plasmid DNAs produced in Escherichia coli TG-1 cells were purified twice on CsCl gradients and verified by DNA sequencing over the amplified regions. The quality and supercoiling of

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**Table**

| Gene* | Subfamily |
|-------|-----------|
| CYP2B | 2B, 2C, 3A |
| CYP2A | 2A, 2C, 3A |

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U67059.

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‡ The abbreviations used are: CYP, cytochrome P450; CAT, chloramphenicol acetyltransferase; 3,3′-DCP, 1,4-bis[2-(3-chloropropyridylo)]benzene; NFI, nuclear factor I; NR, nuclear receptor; PB, phenobarbital; TCPOBOP, 1,4-bis[2-(5,5-dichloropyridylo)]benzene; tk, thymidine kinase; PBREM, PB-responsive enhancer module; bp, base pair; kbp, kilobase pair.
plasmid DNAs were checked by agarose gel electrophoresis.

Preparation, Transfection, and Culture of Mouse Primary Hepatocytes—Two-month-old C57BL/6 males were purchased from Jackson Laboratory (Bar Harbor, MA). About 25×10^6 mouse hepatocytes were electroporated with 30 μg each of individual enhancer/tkCAT reporter plasmids, including 10 μg of pSVβgal control plasmid (Promega) to normalize results between different plasmid DNAs as described previously (17). Equal aliquots from a transfected cell pool were dispensed into four 60-mm dishes to assure identical transfection efficiencies among treatments (17), unattached cells were removed after 30 min, and dishes (about 3×10^6 cells) were incubated with or without inducers for 24 h in Williams’ E-based medium (17) with supplemental 30 mM NaCl, and about 30,000 cpm of 32P-end-labeled oligonucleotide probe. Cell extracts (20 μg) were assayed for protein (21) and pC wt, 5'-GAGGACCAAGACCCAGTCTTTCCTGACCTTGGCACAGTCTatgCATCAACTG-3'.

The free and protein-bound probes were separated on 5% acrylamide DNA fragments or DNA-binding factors are being modified in response to PB or its associated factors was reported (11). Since we found that the −2397/−1850-bp Cyp2b10 DNA fragment mediated PB induction and contains sequences overlapping with the rat 163-bp fragment, we cloned the DNA homologous to the rat 163-bp sequence from the mouse Cyp2b10 gene and found that its identity to CYP2B2 163-bp fragment is 91%, which is higher than the overall 83% identity in 5'–flanking region (17). The corresponding fragment from non-inducible mouse Cyp2b9 gene exhibited a lower 70% identity (Fig. 2A). We then performed DNase I protection assays with the 177-bp Cyp2b10 DNA and identified three weakly protected (pA, pB, and pF) and three strongly protected (pC, pD, and pE) nuclear protein binding regions (Fig. 2B; also see Fig. 2A, bracketed areas). None of these six regions displayed any noticeable differences in binding patterns between control and PB-treated mouse nuclear extracts in gel shift assays (Fig. 2C) or in footprint assays (not shown). These findings suggest that pre-existing DNA-binding factors are being modified in response to PB or that if distinct DNA-binding species activated by PB really exist, they are not detectable by the DNA and oligonucleotide probes used here.

We then examined whether this Cyp2b10 DNA had any PB-inducible enhancer activity in mouse primary hepatocytes. Fig. 3A, top panel, shows that the endogenous CYP2B10 mRNA was strongly increased by PB (lane 2) and TCPOBOP (lane 4) but not by 3,3'-DCP (lane 3), which is an inactive TCPOBOP derivative (17, 26). The mouse albumin mRNA, used as a control, did not respond to the inducers. The same pattern of induction by PB and TCPOBOP (≥ 11-fold) was conferred to the tk promoter by the insertion of 177-bp Cyp2b10 DNA (Fig. 3A, middle panel), whereas the tk promoter alone was not activated by any of the compounds (Fig. 3A, bottom panel). It is notable that the extent of induction was at least as high as with the −2397CAT construct in Fig. 1. Furthermore, the dose responses of the 177-bp Cyp2b10 DNA-driven CAT activity paralleled that of endogenous CYP2B10 mRNA (Fig. 3B). The maximal levels of CYP2B10 mRNA were achieved with 50 nM TCPOBOP and 0.3 mM PB, which induced CAT activity 6.0- and 8.6-fold, respectively. These results indicate that the mouse 177-bp DNA sequence acts as a PB-responsive enhancer with the same chemical specificity and dose-responsiveness as the endogenous Cyp2b10 gene. These data strongly suggest that the 177-bp DNA element mediates PB induction in vivo.

Functional Dissection of the PB-responsive Element—Since there were at least six DNA regions capable of nuclear protein binding within the 177-bp DNA sequence, we next determined their functional role for PB inducibility. Primary hepatocytes were transfected with various DNA deletions linked to tkCAT reporter plasmid (Fig. 4). The deletion of pA had only slight effects on the basal activity or inducibility (compare lanes 1 and 2, and 5 and 7). Depending on the presence of other elements,
the deletion of pE tended to decrease the inducibility by elevating the basal activity about 2-fold at most (compare lanes 1 and 4, and 2 and 6). Due to high basal activity of construct 2364/2250 (lane 3), deletion of pE in this case actually increased the -fold inducibility (compare lanes 3 and 8). The simultaneous removal of pA and pE decreased the inducibility only by 25% (compare lanes 1 and 7). These results suggest that the elements pA and pE do not have a major role in the function of the 177-bp Cyp2b10 enhancer. The removal of pB attenuated the induction response by 40–60%, mostly due to increases in the basal CAT activity (e.g. compare lanes 2 and 3, and 7 and 9). The deletion of pB also attenuated the induction response by 25–50% due to decreases in induced CAT activity but without affecting the basal levels (compare lanes 4 and 5, 6 and 7, and 8 and 9). Finally, the construct -2364/-2297 reproducibly displayed about 3-fold induction indicating that regions pB plus pC harbored the inducer-dependent DNA segment (lane 9).

However, neither pB’ nor pC alone could confer any significant inducibility to the tk promoter (Fig. 4, lanes 10 and 11), suggesting that sequences within both regions are required for induction response. When multimerized DNA fragments were inserted in front of the tk promoter, CAT activity was induced about 2-fold with pC but less with pB’ (lanes 12 and 13). Although the extent of induction was too low to draw a definite conclusion, pC appeared to have a more central role than pB’ in the observed enhancer activity of the pB’-C construct. Consistent with this notion, the most dramatic loss of inducibility (from 6.8- to 1.4-fold) was observed when pC was deleted from the 177-bp Cyp2b10 DNA (lanes 14 and 16). The importance of pB’ for enhancer activity was also confirmed by the fact that deletion of pB’ attenuated the induction to 2.1-fold (lane 15). In addition to these results, we found that the 177-bp Cyp2b10 DNA conferred PB inducibility also to SV40 and proximal Cyp2b10 (-64CAT) (17) promoters, regardless of its orienta-
tion or distance from promoter (data not shown), indicating that the 177-bp Cyp2b10 DNA is a functional enhancer. In summary, our data suggest that pC and pB9 have a major role in determining the inducibility while pB and pD also contribute to the full enhancer activity by modulating the basal and PB-induced activity levels, respectively. Because of this multifactorial nature, we designate the 132-bp Cyp2b10 fragment (22397–2265 bp) as the Phenobarbital Responsive Enhancer Module (PBREM).

The mouse Cyp2b9 gene encodes the female-specific steroid 16α-hydroxylase (18), which is related to Cyp2b10 but not induced by PB (27). The DNA sequence most similar to PBREM was amplified from the Cyp2b9 gene, linked into tkCAT plasmid, and transfected into mouse hepatocytes. As compared with the Cyp2b10 PBREM, the Cyp2b9-derived DNA sequence could not confer any inducibility to the tk promoter-driven CAT activity.

FIG. 3. Specific induction of both endogenous and transfected Cyp2b10 genes. A, top panel, Northern blot analysis of CYP2B10 (upper part) and albumin (lower part) mRNAs from 10 μg of total RNA isolated from about 3 × 10⁶ hepatocytes after 8 h of culture with Me2SO vehicle (lane 1), 1 mM PB (lane 2), 50 mM 3,3′-DCP (lane 3), or 50 mM TCPOBOP (lane 4) as described under “Experimental Procedures.” Middle and bottom panels, about 25 × 10⁶ hepatocytes were electroporated with 30 μg each of 177-bp Cyp2b10-tkCAT (middle) or enhancerless tkCAT (bottom) plus 10 μg of pSVβgal plasmid DNAs. The transfected cells were equally divided into four dishes and incubated with Me2SO vehicle (lane 1), 1 mM PB (lane 2), 50 mM 3,3′-DCP (lane 3), or 50 mM TCPOBOP (lane 4) for 24 h. B, about 25 × 10⁶ hepatocytes were electroporated with 177-bp Cyp2b10 enhancer/tkCAT plasmid and incubated with indicated concentrations of TCPOBOP or PB prior to analysis of mRNA analysis (upper panel) and CAT activity (lower panel) as described in panel A. Cell extracts were assayed for protein, β-galactosidase, and CAT activities as under “Experimental Procedures.” The -fold induction shown are means of normalized CAT activity from two independent transfections with less than 15% difference between experiments. Incubations with tkCAT in bottom part of panel A contained three times more protein to better detect the low CAT activity.

FIG. 4. Functional definition of the PB-responsive enhancer. The brackets and sequence boundaries define the various deletion fragments of the wild-type 177-bp Cyp2b10 enhancer (lane 1) linked into the BamHI site of tkCAT. The plasmids (30 μg) were transfected with pSVβgal plasmid (10 μg) into primary hepatocytes, treated with inducers, and analyzed for protein, β-galactosidase and CAT activities as in Fig. 2. The data shown are normalized CAT activities relative to the wild-type 177-bp Cyp2b10 enhancer (100) without inducer (basal CAT activity) or the ratios of normalized CAT activities between induced and uninduced samples (fold induction) from three independent transfections (mean ± S.D.) unless otherwise indicated.
activity, indicating that Cyp2b10 PBREM displays the predicted genetic specificity of induction (compare PBREM and Cyp2b89 in Fig. 5, A and B). Additionally, the Cyp2b89-driven CAT activity was not increased more than 1.4-fold by TCPOBOP, 3,3′-DCP, or PB (data not shown). Considering these findings and the key role of pC in PBREM function, the sequence of pC was mutated by converting six nucleotides to those in the Cyp2b89 gene while keeping the protected elements pB, pB′, and pD in the Cyp2b10 gene intact. For easier cloning, this was done by first changing the spacer region between pC and pD into an XbaI site, which did not affect the inducibility (PBREM-Xba in Fig. 5, A and B). The subsequent replacement of six nucleotides in Cyp2b10 pC element by corresponding nucleotides in Cyp2b89 gene resulted in the loss of induction of CAT activity (PBREM-mut pC in Fig. 5, A and B), underscoring the importance of pC as the core inducible element within PBREM. The nucleotide sequence of the 33-bp pC fragment contained a perfect nuclear factor I (NFI) binding site (TGGNCCA) and a putative nuclear receptor (NR) binding motif (AGGTCA). Intriguingly, both binding sites are mutated in the noninducible Cyp2b89 gene, the 5′ NFI motif TGG to TGA, and the NR motif AGGTCA to AGaTCA (Fig. 2A).

Functional Analysis of NFI and NR Sites within Element pC—We mutated the NFI and NR binding sites within the minimal inducible fragment pB′-C (−2364/−2297) and examined how these mutations affected binding of nuclear proteins and inducibility of CAT reporter activity. Fig. 6, A and B indicate that nucleotides including NFI and NR motifs between −2326 and −2300 on top strand (upper panel) and between −2333 and −2304 on bottom strand (lower panel) were protected from DNase I digestion in the wild-type pB′-C DNA. Binding to NFI site was dramatically decreased in pC NFI mutant (−2312 GCCCAC to ctatg) so that now only NR motif and seven adjoining nucleotides were protected. In addition, a DNase I hypersensitive site appeared at −2310/−2313, just downstream from the NR site (Fig. 6, A and B, solid arrows). Mutation of the NR site (AGGTCA to AcggA) did not affect NFI binding but somewhat reduced the extent of protection at 5′ end of pC at −2332, −2329, and −2326 (Fig. 6, A and B). Thus, the patterns of nuclear protein binding to pC were altered by NFI and NR mutations, whereas a mutation at pB′ had no effect on protection of pC.

The altered binding patterns were also confirmed in gel shift assays. Previously, we identified a 25-bp DNA element (−1219/−1195) highly similar to a portion of the 163-bp CYP2B2 fragment identified by Trotter et al. (11, 17). This 25-bp probe, here termed PBRE, formed two complexes with liver nuclear extracts (Fig. 6C, lane 1), which could be competed by 50-fold excess of either PBRE or pC oligonucleotides (lanes 2 and 4) but not by oligonucleotides containing mutations at NR motifs (lanes 3 and 5). In line with the DNase I protection assay, mutation of NFI motif considerably reduced binding to pC, and the major remaining complex now comigrated with the top PBRE complex (compare lanes 6 and 8). When both NFI and NR motifs were mutated from the pC probe, this major complex disappeared (compare lanes 6 and 7). In competition experiments, 50-fold excess of PBRE competed for the formation of faster-migrating complexes of pC which were also abolished by mutation of NR motif in pC (lanes 9–11). Finally, the binding to pC NFI mutant harboring an intact NR site could be competed relatively efficiently by PBRE (lanes 12–14). These results indicate that pC and PBRE appear to bind similar factors, that pC can bind both NFI-like and NR-like factors, and the binding of NR-like factors are dependent on the integrity of the AGGTCA motif.

The same mutated DNA fragments used for DNase I protection experiments were used in CAT reporter gene assays (Fig. 7). In five independent transfections, the mutation of NFI binding site reduced the basal CAT activity to 63% and abolished the induction (pC NFIm). The mutation of the NR site increased the basal activity to 221%, but the inducibility was again lost (pC NRm). Mutations of a putative D-binding protein site within pB′ reduced the basal activity but had smaller effects on the induction response (pB′mut1, pB′mut2), confirming the principal role of pC as the core enhancer element. These results are consistent with pC being occupied by at least two factors, NFI- and NR-like proteins. Although both sites are needed to confer inducibility to the minimal pB′-C construct, they appear functionally different, with NFI-like protein acting as an activator and NR-like protein as a repressor, respectively.

**DISCUSSION**

The barbiturate-regulated induction mechanism of bacterial CYP102 gene is well characterized, with a 17-bp so-called Barbie box sequence as the cis-acting element (14, 28). The nature of PB-dependent regulatory elements of mammalian CYP genes, on the other hand, has been very controversial. Some studies indicated that Barbie box-like sequences are present in PB-inducible mammalian gene promoters (at −136/−127 bp in rat α1-acid glycoprotein gene and at −89/−73 bp in CYP2B2 gene) and that they bind nuclear proteins in a PB-dependent manner (13, 14). Similar results were reported with CYP2B2 sequences (−98/−68 bp) overlapping the Barbie box (29). Another group found that the CYP2B2 Barbie box was not protected, and two elements at −199/−183 bp and at −72/−31 bp formed nuclear protein complexes that were more abundant after PB administration (15). However, these studies have relied mainly on in vitro protein binding or in vitro transcription experiments. Only one report showed that mutation of the Barbie box eliminated the 1.6-fold induction by PB of rat α1-acid glycoprotein promoter-driven reporter gene in primary hepatocytes (13).

Several groups have documented that nuclear proteins do not bind to Barbie box-like sequences in α1-acid glycoprotein

**PB-responsive Enhancer Module in Cyp2b10 Gene**
We have now identified, for the first time, the core PB-inducible element that appears to be a classical enhancer and provided some evidence for associated factors. Interestingly, we did not detect any differences in protein binding to PBREM between the control and PB-treated liver nuclear extracts. This implies that PB might act by modifying pre-existing DNA-binding factors, which is consistent with the insensitivity of CYP2B10 mRNA induction to inhibition of protein synthesis by cycloheximide in this hepatocyte system (17). Alternatively, PB might modify the function, affinity, and/or amount of factors associated with the pre-existing DNA-binding proteins. More importantly, we found that the core inducible element is capable of binding NFI- and NR-like factors, both necessary for induction response. We found that NFI and C/EBPα could bind to 32P-labeled pC element in gel shift assays since antibodies raised against these transcription factors were able to supershift some of the pC complexes (data not shown). Our previous studies indicated that another PB-responsive element was located in the –1.4/–1.0-kbp region of Cyp2b10 gene although it could not confer PB-inducibility to heterologous promoters in primary hepatocytes (17). We found, however, that this element contained a 25-bp sequence (PBREM), which was very similar to a portion in the rat 163-bp CYP2B2 gene fragment and contained an AGGTCA motif. We therefore proposed that a NR-like protein may have a role in PB induction of CYP2B2 genes (17). The PBREM also bears similarity to sequences within the present PBREM. It can be aligned with pB (at –2386/–2362 on top strand) but also with pC (at –2333/–2309 on bottom strand). According to our gel shift assays, factors binding to PBREM appear to be related to those occupying the NR site within pC, the DNA region most important for PBREM function.

The PB signal, activating the Cyp2b10 gene, may primarily target the NR-binding (repressor) factor rather than the NFI-like (activator) protein for several reasons. First, NFI isoforms are ubiquitous and present in many tissues and cell lines (35), whereas the Cyp2b10 gene induction is liver-specific (17). Second, NFI binding sites are present, e.g. in mouse albumin (36), rat tyrosine aminotransferase (37), rat CYP2A2 (38), and mouse Cyp2d9 (39) gene promoters, and none of these genes is PB-inducible (17, 40, 38, 27). Third, in our unpublished exper-
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