Phenobarbital Induction Mediated by a Distal CYP2B2 Sequence in Rat Liver Transiently Transfected in Situ*

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The promoter activities of the genes for cytochrome P450 2B1 (CYP2B1) and cytochrome P450 2C1 (CYP2C1) have been assayed by direct injection of promoter-luciferase chimeric genes into rat liver. Activities of minimal promoters for CYP2C1 and CYP2B1 were detectable in untreated animals but were not increased by treatment of the animals with phenobarbital. After insertion to the 5′ side of the minimal promoters of one to three copies of the CYP2B2 sequence from −2318 to −2155, a phenobarbital-responsive element in primary hepatocyte cultures (Trottier, E., Belzil, A., Stoltz, C., and Anderson, A. (1995) Gene (Amst.) 158, 263–268), phenobarbital treatment induced the activity of the CYP2C1 promoter by 5–15-fold and the CYP2B1 promoter by 2.5–5-fold. Mutation of a basal transcription element-like motif and a CCAAT/enhancer binding protein element in the CYP2B1 proximal promoter region reduced expression, but 3–4-fold induction by phenobarbital was retained. Mutation of the ‘Barbie box,’ a putative phenobarbital-responsive element (He, J.-S., and Fulco, A. J. (1991) J. Biol. Chem. 266, 7861–7869) in the CYP2B1 proximal promoter did not reduce the relative response to phenobarbital. These results demonstrate that direct injection of DNA into rat liver may be used to assay phenobarbital responsiveness of cytochrome P450 genes. In this system, a distal CYP2B2 element mediates a response to phenobarbital, and proximal elements, including the Barbie box, are not required for the induction.

The induction of drug-metabolizing enzymes by phenobarbital (PB)† was reported almost 40 years ago (1). Cytochromes P450 are the primary enzymes responsible for phase I metabolism of drugs, and PB has been shown to increase the amounts of P450 enzyme and mRNA by stimulation of gene expression (2). PB-responsive P450 genes have been isolated and characterized, but progress in determining the molecular mechanism by which this drug activates gene expression has been impeded by the lack of a continuous cell line that is competent for PB induction. Transcription in cell-free systems of a CYP2B1/2 mini-gene containing 179 bp of the 5′-flanking region was induced in liver extracts from PB-treated rats (3, 4), and this same sequence responded to PB when introduced into rat liver cells in vivo as a complex with asialo glycoproteins (5). PB-dependent changes in binding of nuclear proteins to proximal elements have been detected by gel shift and DNase I footprinting assays and have led to a model in which positive and negative regulatory elements are involved in the PB response (5). In other studies, a sequence motif was identified as a PB-responsive element (‘Barbie box’) in CYP genes in Bacillus megaterium, and similar motifs were found in other PB-responsive genes in several species including the proximal promoter of mammalian CYP2B1/2 and CYP2C1 (6–8). Proteins in extracts from B. megaterium and rat liver nuclei bound to these sequences in the bacterial and mammalian genes in a barbiturate-dependent manner (6). Mutation of a Barbie box in the rat α1-acid glycoprotein promoter eliminated a 1.6-fold induction by PB in transfected hepatocytes in primary culture (9).

In contrast to these studies implicating a proximal promoter sequence as a PB-responsive element, other studies have suggested that elements 5′ distal to the promoter mediate PB induction. A distal enhancer-like element in the chick CYP2H1 gene was reported to mediate PB induction of a heterologous promoter in primary cultures of chicken hepatocytes (10). Transgenes in mice, which contained the CYP2B2 gene with 800 bp of 5′-flanking region including the proximal promoter elements discussed above, were expressed constitutively at high levels and were not induced by PB, while expression of transgenes containing 19 kb of 5′-flanking region was dependent on PB treatment (11). Consistent with this result a CYP2B2 sequence from −2318 to −2155 was shown to mediate PB induction in primary rat hepatocyte cultures with its homologous promoter and a heterologous promoter (12). In studies on the mouse Cyp2b10 gene, deletion of sequences from −1404 to −971 resulted in elimination of a 2–3-fold response to PB in transfected primary hepatocytes (13). Interestingly, this region in Cyp2b10 is highly similar to the corresponding region in CYP2B2, but the constructions of the CYP2B2 promoter in which the −2318 to −2155 region were deleted, but the sequences similar to the −1404 to −971 region in the mouse gene were retained, were not induced by PB in transfected primary hepatocytes (12). These results indicate that the proximal promoter alone in CYP2B2 genes is not sufficient for PB induction, but that distal elements are also required.

We now report that direct injection of DNA into rat liver is an effective in situ transient transfection method to assay CYP promoter activity. The distal PBRE reported by Trottier et al. (12) mediates PB responsiveness in this system and mutation of proximal promoter elements, including the Barbie box, does not eliminate the response.

EXPERIMENTAL PROCEDURES

Oligonucleotides—The following oligonucleotides were synthesized on an Applied Biosystems model 380A DNA synthesizer at the Biotech-
Phenobarbital Induction of CYP2B2

To confirm the role of the PBRE in PB induction, single, double, and triple copies of the PBRE were inserted into EcoRI-BamHI-digested plasmid pTZC1–272. Orientation of single, double, and triple copies of the PBRE in pTZC1–272 was identified by sequencing. The hybrid PBRE-C1–272 fragments were excised by digestion with SmaI and HincIII and inserted into the promoterless luciferase vector pαLUC (19) or MspI and with the same enzymes and with a 3′-flanking region fused to a luciferase reporter gene in rat liver (Fig. 1). The open boxes within the proximal promoter regions represent the proximal promoter constructs were Cloned in the natural orientation these CYP2C1 constructions were amplified by PCR using a T7 promoter primer and primer 2C1–257. The PCR products were digested with EcoRI and BamHI and inserted into EcoRI-BamHI-digested wild type pTZB1–110 or its BTE and C/EBP constructs; however, the orientation of the copies in the CYP2C1 construction was reversed. To obtain the proper orientation, the EcoRI-BamHI-digested PCR product was inserted into EcoRI-BamHI-digested pTZ15R, reexcised by digestion with SmaI and HincIII and cloned into the Smal site of pTZB1–110. The correct orientation was confirmed by sequencing. The Barbie box was mutated by PCR using BBM1 or BBM2 as a 3′-primer, 2BA as a 5′-primer and pB1–110LUC as a template. Mutated PCR products were digested with BamHI and HincIII, inserted into pTZ15R and to form pTZBBM1 and pTZBBM2, and three copies of the PBRE were inserted into these vectors as described above for a CYP2C1 construct that increased expression of the injected gene (20). Shortly after rats recovered from surgery, 1 mg of dexamethasone/kg was injected subcutaneously 24 h before surgery as an anti-inflammatory agent that increases expression of the injected gene (20). Shortly after rats recovered from surgery, 1 mg of dexamethasone/kg, subcutaneously, and either saline or 100 mg of PB/kg, intraperitoneally, were injected. Rats were sacrificed 24 h later to obtain liver tissues for luciferase assay.

Luciferase Assays—Luciferase assays were performed as described with minor modifications (21). Rats were killed by ether or CO2 overdose, and liver tissue containing the sites of injection was excised and homogenized in 1.5 ml of 1% Triton X-100, 25 mm glycyglycine, pH 7.8, 15 mm MgSO4, 4 mm EGTA, 1 mm dithiothreitol, 0.1 mm phenylmethylsulfonyl fluoride, and 0.5 μg/ml leupeptin in a Dounce tissue grinder with 20 strokes with a tight pestle. The homogenates were centrifuged for 20 min at 4 °C in a microcentrifuge (Beckman Instruments). Supernatants were then transferred to fresh 1.5 ml Eppendorf tubes, and 0.1 ml was used for each luciferase assay. A background of 300 arbitrary light units and for reference, the means of measured activities for the control and PB-treated samples was 1782 and 1325, respectively, for the CYP2B1 construction without a PBRE. Animals were treated with saline (solid filled bars) or with 100 mg PB/kg (stippled bars). The numbers of rats injected in each treatment group are shown at the tops of the bars in parentheses, the fold induction by PB is indicated, and the standard error of the mean is shown. The assays for CYP2B1 promoter constructions with the single and double copies of the PBRE were done several months after the rest of the assays and resulted in higher activities for the untreated animals than in the earlier studies for unknown reasons. Activities of PB-treated groups were significantly different from the corresponding untreated groups (p < 0.05, Student’s t test) for each construction containing the PBRE except for BBM2.

RESULTS

Mediation of PB Induction by a Distal Sequence in Rat Liver Transfected in Situ—Injection of DNA of chimeric genes containing −3500 (data not shown) or −272 bp of CYP2C1 5′-flanking region fused to a luciferase reporter gene into rat liver resulted in a low level of expression of luciferase about 2-fold greater than that in liver injected with pαLUC, a promoterless vector (Fig. 1A). Treatment of rats with 100 mg of PB/kg for 24 h before sacrifice did not increase activity for the CYP2C1 construction. To determine if the CYP2B2 sequence from −2318 to −2155 (PBRE) (12) could confer PB responsiveness to the CYP2B1 promoter, a single copy or three copies of this sequence was inserted 5′ of the CYP2B1 promoter. Insertion of the PBRE had little effect on CYP2C1 the promoters in the CYP2B1 constructions without a PBRE. Animals were treated with saline (solid filled bars) or with 100 mg PB/kg (stippled bars).

Luciferase Assays—Luciferase assays were performed as described with minor modifications (21). Rats were killed by ether or CO2 overdose, and liver tissue containing the sites of injection was excised and homogenized in 1.5 ml of 1% Triton X-100, 25 mm glycyglycine, pH 7.8, 15 mm MgSO4, 4 mm EGTA, 1 mm dithiothreitol, 0.1 mm phenylmethylsulfonyl fluoride, and 0.5 μg/ml leupeptin in a Dounce tissue grinder with 20 strokes with a tight pestle. The homogenates were centrifuged for 20 min at 4 °C in a microcentrifuge (Beckman Instruments). Supernatants were then transferred to fresh 1.5 ml Eppendorf tubes, and 0.1 ml was used for each luciferase assay. A background of 300 arbitrary light units, observed in liver extracts after pαLUC injection, was subtracted, and total activity was determined based on the total volume of the supernatant. Statistical significance between the untreated and PB-treated samples was tested by one-tailed Student’s t test.
than normally observed, and the difference between the PB-treated and untreated groups was not statistically significant in this one case (Fig. 1B). The similar results with two mutations indicate that the small increase in activity is due to the mutated Barbie box and not to inadvertent creation of a new positive regulatory site. The continued PB response with each of the BTE, C/EBP, and Barbie box mutations suggests that the PBRE is a PB-dependent enhancer and not a general enhancer dependent on proximal promoter elements for PB responsiveness.

**DISCUSSION**

A major impediment to analysis of the genetic mechanisms involved in the PB induction of cytochrome P450 and other drug-metabolizing enzymes has been the lack of a continuous cell culture model system in which promoter activity could be assayed. Primary cultures of hepatocytes (12, 13), cell-free transcription (3, 4, 17, 22, 23), and targeting to the liver of DNA complexed to asialoglycoproteins (5) have been reported as methods in which PB responsiveness is retained. These methods are relatively complex, and the responsiveness in cell-free systems has been very modest compared to the induction observed in vivo. DNA injected directly into rat liver has been shown to be taken up by hepatocytes and expressed (20), and as reported here, is a relatively simple, reproducible method for analysis of PB responsiveness of CYP genes. With the luciferase reporter, results can be obtained less than 2 days after the injection of the DNA, and the range of variability is usually about 2-fold, so that changes of 4–15-fold as observed here are easily detected with small numbers of animals in each treatment group. There are two shortcomings of this system at present. The uptake and expression of DNA is inefficient so that 300–500 μg must be injected in each liver. The low efficiency has precluded the used of an internal standard to allow normalization of the efficiency of uptake and expression of the injected DNA, since only the luciferase assay has sufficient sensitivity for detection. Sensitive chemiluminescent assays of β-galactosidase (24) may provide a second reporter that could be used for normalization, and this possibility is being examined. The second disadvantage is that dexamethasone treatment is required to increase the efficiency of expression of the injected genes by the liver (20), which might alter the response to other ligands, such as PB. Even with these disadvantages, the simplicity and speed of the assay should permit rapid identification of the elements required for PB induction in CYP and other drug metabolizing enzyme genes.

These *in situ* transfection experiments provide strong support for the role in PB-dependent gene expression of the distal PBRE identified by transfections of primary hepatocyte cultures (12) and are consistent with CYP2B2 transgenic studies (11) which indicated that sequences distal to −800 were required for PB induction. Induction of up to 15-fold with triple copies of the PBRE and 2.5-fold with single copies of the PBRE were observed which are less than the 20–100-fold increases of mRNA levels observed for CYP2B2 and CYP2B1 *in vivo* (25–27). The low induction may be due to the relatively high expression observed in the injected liver in untreated animals, particularly for CYP2B1, since expression of this gene *in vivo* is not detectable in untreated animals (28). Sequences which mediate the inhibition *in vivo* might be missing from the constructions or normal chromatin structure may be required for the suppression which is lacking in genes transiently transfected. The PBRE was able to confer PB induction on minimal promoters of its homologous gene as well as a heterologous gene, CYP2C11, as assayed by injection in *situ*. In primary hepatocyte cultures, this sequence conferred PB responsiveness to the heterologous herpes simplex thymidine kinase gene (12). In the hepatic direct injection assay, like the primary
hepatocyte culture (12), the induction was orientation-independent (data not shown), and induction was proportional to increased copies of the PBRE, characteristics common to enhancer sequences. The ability of this element to confer PB responsiveness to heterologous promoters and the continued responsiveness when proximal promoter sequences are mutated establish that this upstream enhancer-like element is a PB-responsive element. The demonstration that this distal region is hypersensitive to DNase I treatment of intact chromatin and that the relative sensitivity is altered by PB treatment provide further support for the regulatory role of this region (29).

In contrast to the distal elements, the in situ transfection experiments do not provide support for a role of the proximal promoter region of CYP2B1 and CYP2C1 in PB induction. It has been suggested that upstream elements might be nonspecific enhancers, with PB regulation exerted at the proximal promoter region (5). However, in the absence of the PBRE, promoter fragments from –3500 to +1 (data not shown), –272 to +1 for CYP2C1, –1400 to +1 (data not shown), and –110 to +1 for CYP2B1 did not exhibit any positive response to PB treatment. Likewise, PB induction was not observed in transfected primary hepatocytes which contained CYP2B2 promoter fragments from –2015 or –1680 to 1 (12). Further, mutation of the known positive regulatory elements in the CYP2B1 promoter (15) and the proposed PB-responsive element, Barbie box (8), did not substantially alter the relative induction by PB. Mutation of the BTE and the C/EBP reduced activity 65 and 80%, respectively, in untreated animals, which is similar to the reduction in activity of 70–75% observed for these mutations in HepG2 cells (15). Interestingly, the Barbie box mutations increased activity 2–3-fold, suggesting that this element might have modest negative regulatory activity. Studies with the mouse Cyp2b10 gene, which is 83% similar in the 5’-flanking region to CYP2B1, transfected in primary hepatocytes, also indicated that distal elements mediate PB induction (13). In this mouse gene the Barbie box is split by a 42-bp insertion, and this lack of motif conservation suggests that the Barbie box sequence is not a common PB element in P450 genes.

It is possible that PB responsiveness of proximal elements is not detected in the in situ assays either because the assay is not sensitive enough or because of the conditions of the assay compared to other assays. In some cell-free studies, PB responsiveness was modest (17), less than 2-fold, and this level of induction might be difficult to detect in the in situ injection assay in which induction mediated by the PBRE was considerably less than induction of CYP2B1 in vivo. However, 8-fold and higher induction with only 179 nucleotides of CYP2B2 5’-flanking region was observed in other cell-free studies and when DNA was targeted to the liver as a complex with asialoglycoproteins (3, 5). The reason for the different results observed with the in situ injection assay is not immediately obvious. A second caveat is that dexamethasone treatment is required for successful in situ transfection, and dexamethasone has been reported to both increase or decrease PB induction of P450 genes (2). Dexamethasone has been reported to reduce PB induction mediated by the 179-bp CYP2B1 promoter fragment (4, 30). Earlier studies (30) used doses of dexamethasone 10–50-fold higher than the 1 mg/kg used in these in situ injection experiments, but the more recent studies (4) reported partial inhibition of PB induction by 0.1 mg of dexamethasone/kg. However, in transgenic mice, dexamethasone is not required, and therefore the lack of PB responsiveness of CYP2B2 transgenes containing 800 bp of 5’-flanking sequence (11) argues against dexamethasone masking of PB-proximal elements as an explanation for their lack of PB responsiveness in the in situ injection assay. The data with the in situ injection assay show that the proximal elements are not required for the PB induction mediated by the distal elements and are most consistent with a major role for the 5’ distal PBRE as a PB-response element and a modest role, if any, for proximal promoter sequences.

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