The CYP2B2 Phenobarbital Response Unit Contains an Accessory Factor Element and a Putative Glucocorticoid Response Element Essential for Confering Maximal Phenobarbital Responsiveness*

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Hepatic cytochrome P450s play a critical role in the metabolism of hydrophobic xenobiotics. One of the major unsolved problems in xenobiotic metabolism is the molecular mechanism whereby phenobarbital induces hepatic enzymes, particularly CYP2B1 and CYP2B2 in rat liver. By using primary rat hepatocytes for transfection analyses, we previously identified in the CYP2B2 5′-flank a 163-base pair Sau3AI fragment that confers phenobarbital inducibility on a cat reporter gene and that has the properties of a transcriptional enhancer. Transfection experiments with sub-regions of the Sau3AI fragment now indicate that a central core together with an upstream or downstream accessory element within the fragment can confer phenobarbital responsiveness. One such accessory element, AF1, was identified and localized. DNase I footprinting analysis revealed the presence of a footprint overlapping this AF1 element. It also identified three other major protected regions, two of which are putative recognition sites for known transcription factors. Site-directed mutagenesis indicated that a putative glucocorticoid response element as well as a nuclear factor 1 site and an associated nuclear receptor hexamer half-site are essential for conferring maximal phenobarbital inducibility. Taken together, the results indicate that phenobarbital induction of CYP2B2 requires interactions among multiple regulatory proteins and cis-acting elements constituting a phenobarbital response unit.

Many different cytochrome P450s (CYPs) are involved in the hepatic metabolism of a wide variety of xenobiotic substances including drugs, plant metabolites, and chemical carcinogens (1, 2). The genes encoding these enzymes are either expressed constitutively or are induced by various chemicals (3). One of the major unsolved problems in the study of the induction of CYP proteins is the molecular mechanism whereby phenobarbital (PB) induces the closely related CYP2B1 and CYP2B2 forms in rat liver (4). Although it has long been known that PB induces CYP2B1 and CYP2B2 mRNAs by increasing transcription of their genes (5, 6), details of the transcriptional control of CYP2B1 and CYP2B2 have not been forthcoming. This is largely because, until recently, it was not possible to obtain PB induction of the endogenous CYP2B1 and CYP2B2 genes in cultured cells (4, 7, 8). We have transfected reporter gene constructs into cultured adult rat hepatocytes and localized, in the CYP2B2 5′-flanking region, a 163-base pair (bp) Sau3AI fragment that confers PB inducibility on a cat reporter gene (9). The Sau3AI fragment, which is situated between 2155 and 2317 bp upstream of the CYP2B2 transcription start point, in the vicinity of a liver-specific DNAse I-hypersensitive site (10), has the properties of a transcriptional enhancer (9). The capacity of the Sau3AI fragment to confer PB responsiveness on a heterologous promoter has been confirmed in a quite different assay system involving in situ DNA injection into rat liver (11). Furthermore, the homologous region of the 5′-flanking region of the PB-inducible mouse Cyp2b10 gene contains a segment 91% identical to the rat CYP2B2 163-bp fragment that also confers PB inducibility on heterologous promoters and possesses the properties of a transcriptional enhancer (12).

The CYP2B2 163-bp Sau3AI fragment contains a functional nuclear factor 1 (NF1) site (9, 13) and recognition sites for other sequence-specific DNA binding factors present in rat liver nuclear extracts (9). In the present study, we sought to define the elements within the 163-bp Sau3AI fragment which confer PB responsiveness. Deletion constructs of the Sau3AI fragment, as well as constructs in which putative recognition sites for DNA binding factors had been mutated, were transfected into adult rat hepatocytes, and their effect in conferring PB responsiveness was analyzed. DNase I footprinting experiments were used to identify potential regulatory elements by defining interactions between rat liver nuclear proteins and the Sau3AI fragment. A putative glucocorticoid response element as well as an NF1 site and an associated nuclear receptor hexamer half-site were found to be essential for conferring maximal phenobarbital inducibility. Taken together, the results indicate that the Sau3AI fragment is a multicomponent enhancer and that multiple regulatory proteins and their cognate recognition sequences within it are critical for obtaining maximal PB responsiveness. Thus, the Sau3AI fragment constitutes a PB response unit (PBPU), analogous to the complex glucocorticoid response unit (GRU) required for glucocorticoid induction of transcription of the rat gene for phosphoenolpyruvate carboxykinase (PEPCK) (14–17).
CYP2B2 Phenobarbital Response Unit

EXPERIMENTAL PROCEDURES

Materials and Animals—Chee's medium for hepatocyte culture as well as restriction and DNA-modifying enzymes were from Life Technologies, Inc. The double-stranded CfT/CF1 consensus oligodeoxyribonucleotide (oligo) (5'-CTCTTTGACGATGCTGGCAATTG-3') was from Promega or was purchased as complementary single-stranded oligos from Life Technologies. Other oligos were also from Life Technologies, except for those noted. [α-32P]dTTP (6000 Ci/mmol), [32P]dATP (3000 Ci/ mmol), [α-32P]dCTP (1250 Ci/mmol), [32P]dGTP (1450 Ci/mmol), and [32P]dTTP (1450 Ci/mmol) were from NEN Life Science Products. Male Sprague-Dawley rats (150–180 g) were from Charles River Canada.

Isolation and Culture of Primary Hepatocytes, Transfection, PB Treatment, and Chloramphenicol Acetyltransferase (CAT) Assays—Methods for hepatic isolation and culture, essentially that of Waxman et al. (18), as well as those for liposome-mediated transfection (19) and PB treatment have been described (9). Plasmids were purified using a plasmid purification kit (Qiagen). CAT activity was assayed by the method of Gorman et al. (20) and, occasionally, by that of Seed and Shen (21).

Construction of Deletion and Point Mutants—Sequential 5' or 3' deletions of the 163-bp Sau3AI fragment (2157–2155) generated by restriction enzyme or Bal31 digestion (Fig. 1). The starting point for construction of such deletion mutants was the pSaA163 plasmid, obtained by subcloning the 163-bp Sau3AI fragment into the Smal site of pBluescript KS1 (Stratagene). The pSaA163 plasmid or similar pBluescript KS derivatives containing specified portions of the CYP2B2 5'-flank were used to generate other deletions by polymerase chain reaction-mediated amplification. To obtain deletions of the 163-bp Sau3AI fragment, obtained by subcloning the 163-bp Sau3AI fragment to the RV site of the non-PB responsive Ev construct which contains the normal orientation and 35S-l3AI fragment (already carried NF1 m1), it generated a 3AI fragment using the method of Gorman et al. (20) and, occasionally, by that of Seed and Shen (21).

RESULTS

Functional Analysis of the 163-bp Sau3AI Fragment—Transfection analysis was performed with subfragments of the 163-bp Sau3AI fragment to identify sequences within it that might confer PB responsiveness. Sequential deletions from the 5' end up to the internal NcoI site (coordinate −2257) were all active in conferring PB responsiveness, although the response conferred by construct −2257/−2155 was reduced by about 2-fold as compared with that of the full-length fragment (Fig. 1A, constructs with a common 3' end point of −2155 and 5' end points of −2290, −2283, −2273, −2264, or −2257). Further deletion from the 5' end, to an internal RsaI site (coordinate −2230) or to −2180, led to complete loss of activity (Fig. 1A). Similarly, sequential 3' deletions up to −2207 were also active, although the response conferred by constructs −2137/−2188 and −2217/−2207 was again reduced by about 2-fold as compared with that of the full-length fragment (Fig. 1A, constructs with a common 5' end point of −2217 and 3' end points of −2172, −2188, or −2207). Further deletion from the 3' end, to the RsaI site (coordinate −2231; Fig. 1A) or to −2253 (coordinate −2600/−2253; data not shown), led to complete loss of activity.

These results suggested that DNA sequence elements that are essential for PB responsiveness are localized in the 51-bp central core between −2257 and −2207. However, construct −2257/−2208 was completely inactive (Fig. 1), although its 5' extremity is the same as that of the active −2257/−2155 construct and its 3' extremity is 1 bp upstream of that of the active −2231/−2207 construct. Since construct −2257/−2207 was also inactive (Fig. 1B), the single base pair difference at the 3' ends of the −2257/−2208 and −2317/−2207 constructs does not account for the inactivity of the former. Hence, the central core is insufficient by itself to elicit PB responsiveness.

Functional Evidence for an Accessory Element—The deletion analysis presented above suggests the following model to account for the PB responsiveness conferred by the 163-bp Sau3AI fragment. A central core, together with an element or elements extending upstream of coordinate −2257 or with an element or elements extending downstream of coordinate −2207, can confer PB responsiveness. In support of this model, although construct −2257/−2188 was inactive, construct −2257/−2172 was active in eliciting a PB response (Fig. 1B). This result indicated that there is a DNA sequence element between coordinates −2188 and −2172 that confers PB responsiveness when combined with the central core. The element between −2188 and −2172 is insufficient to elicit PB responsiveness by itself; because it is present in the inactive −2320/−2155 construct (Fig. 1A). Hence, it is an accessory site for conferring PB responsiveness, and we have designated it as AF1 (Figs. 1B and 2).

Physical Evidence for Proteins Binding to AF1 and to Other Sites within the 163-bp Sau3AI Fragment—To ascertain whether an accessory factor binds to the AF1 site, as well as to look for evidence of other protein-DNA interactions, the 163-bp Sau3AI fragment was subjected to DNase I footprinting analysis using rat liver nuclear extracts. First, the fragment was labeled on the lower strand at the 5' end (Fig. 3A). Analysis using crude rat extract revealed a series of almost continuous footprinting over 85 bp extending from about −2255 to about −2200 (Fig. 3A). Use of heparin-Sepharose-fractionated extracts facilitated resolution of protected regions F1, F2, F3, and F4 within this segment: protected region F2 was virtually undetectable with the fractionated extracts, whereas F1, F3 and F4 remained visible (Fig. 3A). An additional protected region, F0, was revealed after 5' end-labeling of the lower
strand (Fig. 3B). F0 is also visible above the F1 footprint in Fig.
3A (crude extract lane). Protected regions F1', F2', and F3',
corresponding to F1, F2, and F3 plus F4, respectively, were
identified by labeling the 5' end of the upper strand (Fig. 3C).
Protected region F0' (corresponding to F0) is also evident above
F1' in Fig. 3C. The positions of the F0 and F0' footprints overlap
with the AF1 site defined by transfection analysis (Fig. 2).

Addition of PB to incubation mixtures did not appreciably
change the footprints obtained with crude nuclear extracts of
untreated rats (data not shown) or with a nuclear extract
partially purified by heparin-Sepharose fractionation (Fig. 3A,
lanes PB), and similar footprints were generated by crude
nuclear extracts from untreated and PB-treated rats (Fig. 3B
and data not shown).

The 163-bp CYP2B2 Sau3AI Fragment Is a Multicomponent Enhancer—The DNA sequence of the 163-bp Sau3AI fragment
(13) (see also Fig. 2) reveals that it contains putative recognition
sites for several transcription factors, only some of which
are shown in Fig. 2. Potential regulatory motifs were identified
by inspection and by application of MatInspector (matrix simi-
larity threshold, 0.85), a search tool for scanning DNA se-
quences for matches to nucleotide distribution matrices for
transcription factor binding sites accessible in the TRANSFAC
data base (24). Immediately adjacent to a perfectly symmetri-
cal NF1 site (−2217 TGGN7CCA) (25), previously identified (9,
Fig. 2. DNA sequence of the CYP2B2 163-bp Sau3AI fragment. The DNA sequence is from Hoffmann et al. (13), except that our cloned sequence has three not four T residues immediately 5' of nucleotide −2225 (vertical arrowhead). The sequence of the CYP2B2 fragment is identical to that of the corresponding CYP2B1 fragment (44) except for a single base difference at CYP2B2 nucleotide −2299 (asterisk), which is C in CYP2B2 and G in CYP2B1. Protected regions on the upper and lower strands are shown by upper and lower brackets, respectively. The Sau3AI restriction sites and the internal NcoI site are boxed. Nuclear receptor hexamer half-sites in the ER-7 arrangement and forming part of the HX-NF1 complex are identified by horizontal arrows; the NF1 site is overlined, and the sequence of the AF1 site is shaded. Candidate glucocorticoid receptor binding sequences on the upper and lower strands are denoted by a dotted overline and a dotted underline, respectively. The central core sequence between −2257 and −2207 is delimited by the region between the two vertical lines. Footprint coordinates on the lower strand are as follows: F0, −2171 to −2180; F1, −2202 to −2234; F2, −2239 to −2251; F3, −2261 to −2271; F4, −2272 to −2285. Footprint coordinates on the upper strand are: F0', −2171 to −2184; F1', −2191 to −2229; F2', −2242 to −2257; F3', −2262 to −2283.

The F1 and F1' footprints extend from −2230 to −2192, a region which includes and extends beyond the HX-NF1 complex (Fig. 2). Most of the F1 footprint was eliminated by a CTF/NF1 consensus oligo competitor (Fig. 3A, lane NF1). Therefore, it is caused in part at least by an NF1 protein. The positions of F3/F4 and F3' correspond to the two halves of the ER-7 sites, and both F3 and F4 were competed by oligo HX (Fig. 3A, lane HX). Hence F3/F4 and F3' are presumably caused by protein(s) of the nuclear receptor superfamily binding to the two halves of ER-7. No good candidates for the protein(s) responsible for the F2 and F2' footprints are yet available.

The region of the F1' footprint was analyzed using varying amounts of nuclear protein. At lower protein levels, a reduced protected region was observed with the wild-type sequence, presumably as a result of binding of an NF1 protein, whereas at higher protein levels, the footprint was extended in both directions (Fig. 4A). Similar results were obtained with protein extracts from untreated and from PB-treated rats (Fig. 4A). When a mutant sequence in which NF1 binding was abolished was analyzed, the extended protection on the upstream side was still evident at higher protein levels (Fig. 4B). Thus, the extended protection on the upstream side is due to binding of a protein or proteins to a sequence including the nuclear receptor hexamer half-site. Consistent with this conclusion is the observation that only a part of the F1 (Figs. 3A and 5A) and the F1' (Fig. 5B) footprints were eliminated by competition with an NF1 consensus oligo.

PB Responsiveness is Reduced but Not Eliminated by Mutating Either or Both Elements of the HX-NF1 Complex—To investigate the role of the HX-NF1 complex in conferring PB responsiveness, the NF1 site was mutated in two steps as follows: first in the distal portion (NF1m1; Fig. 6) and then in both the distal and proximal portions (NF1dm2; Fig. 6). The NF1 m1 mutation created a new DNase I-hypersensitive site at −2207 and modified the F1 footprint but did not abolish it; furthermore, the portion of the modified F1 footprint corresponding to NF1 sites was eliminated by competition with an NF1 consensus oligo (Fig. 5A). With the NF1dm2 mutant sequence, the F1' footprint was reduced to that seen with the wild-type sequence in the presence of an NF1 consensus oligo competitor (Fig. 5B). Hence, the NF1dm2 mutation completely eliminated detectable NF1 binding, but it left a footprint on the upstream side, corresponding to the anticipated binding of a protein or proteins to a sequence including the nuclear receptor hexamer half-site (Figs. 4B and 5B). Here, as elsewhere, identical results were obtained using nuclear extracts prepared from livers of untreated and PB-treated rats (Fig. 5B).

The NF1 m1 and NF1dm2 mutations, as well as two additional mutations of the HX-NF1 complex, HXm (mutated in the hexamer half-site) and HXm-NF1dm2 (mutated in the hexamer half-site and in NF1), all reduced but did not abolish PB responsiveness when tested by transfection analysis (Fig. 6). None of these mutations increased the basal level of CAT activity (data not shown). These results demonstrated that both elements of the HX-NF1 complex must be intact to elicit a maximal response to PB.

Mutation of a Candidate Glucocorticoid Receptor Binding Site Dramatically Reduces PB Responsiveness—The consensus sequence for the glucocorticoid response element (GRE) is GG-TACAnnnTGTTTC (28). The two candidate glucocorticoid receptor binding sites found by MatInspector within the central core defined by transfection analysis contain putative GREs, one of which, −2244 GGCACAgacTCTGTA on the upper
strand, matches the consensus at 7 of 12 positions. This sequence was mutated to GGCGTGgacTCTGTA, thereby reducing the match to 4 of 12. This led to virtual abolition of the PB response (Fig. 6). This suggests that a protein binding in the region of the putative GRE is required to confer PB responsiveness.

Mutation of the CYP2B2 Barbie Box Does Not Affect PB Inducibility—It has been suggested that the Barbie box (29) is involved in conferring PB responsiveness on the rat CYP2B1 and CYP2B2 genes (30). However, when the core sequence (5’-AAAG-3’) of the CYP2B2 Barbie box was mutated, PB responsiveness was retained (Fig. 6).

**DISCUSSION**

Our previous work has shown that an upstream enhancer element, located between −2317 and −2155 in 5′-flanking region, confers PB responsiveness on the rat CYP2B2 gene (9). This element is situated in the vicinity of a liver-specific DNase I-hypersensitive site in chromatin (10). Such sites are a hallmark of regulatory regions (31). Furthermore, in transgenic mice a rat CYP2B2 transgene including only the first 800 bp of the 5′-flank was not PB-inducible, whereas a transgene carrying 19 kb of 5′-flank (and hence the −2317/−2155 segment) was normally inducible (32). The experiments described in our previous report (9) and in subsequent reports from two other laboratories (11, 12), as well as those described here, provide a strong body of evidence indicating that upstream control elements confer PB responsiveness on the rat CYP2B2 and mouse Cyp2b10 genes. The mouse Cyp2b10 active sequences are situated between −2426 and −2250 and display 91% sequence identity to those of CYP2B2 (12). Particularly important in this context is the observation that generally similar results have been obtained in three different laboratories using different experimental approaches and PB-inducible CYP2B genes of two different rodent species.

The results presented here, showing that at least three and probably more sequence elements within the 163-bp Sau3AI fragment are required to confer maximal PB responsiveness,
reveal the surprising complexity of this multicomponent enhancer. According to the now classical model for steroid hormone action, the liganded receptor binds to one or more response elements upstream of the target gene and acts as a transcriptional enhancer (26). However, synergistic interaction of a variety of transcription factors with steroid hormone response elements have been known for some time (33), and in a number of genes several cis-acting elements function in concert to activate transcription (34). The best studied such case is that of the rat PEPCK gene. Full glucocorticoid induction of PEPCK transcription is mediated by a complex GRU consisting of two glucocorticoid response elements, as well as three accessory factor binding sites (14, 17). We originally referred to the CYP2B2 Sau3AI fragment as a PB-responsive element (9), but, in the light of its complexity, we now refer to it as a PBRU (35, 49). The homologous mouse Cyp2b10 sequence has been termed a module (12, 36). Proteins that can serve as accessory factors in the PEPCK glucocorticoid response include hepatocyte nuclear factor-3 (16) and the orphan receptors chicken ovalbumin upstream promoter transcription factor (15, 17) and hepatocyte nuclear factor-4 (HNF-4) (15). The application of the PEPCK GRU model to PB regulation of CYP2B2 does not imply that any particular recognition site is shared between the two response units, but merely that PB induction of CYP2B2 requires interactions among multiple regulatory proteins and cis-acting elements constituting a PBRU.

Fig. 4. Analysis of the F1' footprint with various amounts of protein. DNase I footprinting assays were performed using unfractionated rat liver nuclear extract. The fragment was 5' end-labeled on the upper strand. Only the region of the F1' footprint is shown. BSA denotes DNase I treatment in the absence of nuclear proteins, and control and PB denote DNase I treatment with nuclear extracts from untreated and PB-treated rats, respectively. Brackets labeled NF1 and HX denote protected regions caused by proteins binding to the NF1 site and to a sequence including the nuclear receptor hexamer half-site, respectively. A, the wild-type fragment was used. Incubations were performed in the presence of 20 mM sodium β-glycerophosphate, and the amounts of nuclear extract protein per assay were 2.5, 5, 7.5, 10, 25, 50, 75, and 100 µg. B, the fragment carrying the NF1dm2 mutation was used. Incubations were performed in the presence of 10 mM sodium β-glycerophosphate, and the amounts of nuclear extract protein per assay were 2.5, 5, 7.5, 10, 25, 50, and 75 µg.

Fig. 5. Effect of mutations in the NF1 site on the F1 and F1' footprints. A, the wild-type fragment or the fragment carrying the NF1 m1 mutation was treated with unfractionated nuclear extract (100 µg of protein) from livers of untreated rats. Fragments were 5' end-labeled on the lower strand. NF1 denotes the presence of a 500-fold molar excess of the NF1 consensus oligo competitor. The position of the F1 footprint is shown by a box, and HS at coordinate −2207 denotes a hypersensitive site. B, the wild-type fragment or the fragment carrying the NF1dm2 mutation was used. Fragments were 5' end-labeled on the upper strand. BSA denotes DNase I treatment in the absence of nuclear proteins, and control and PB denote DNase I treatment with nuclear extracts (100 µg of protein) from untreated and PB-treated rats, respectively. NF1 denotes the presence of a 500-fold molar excess of the NF1 consensus oligo competitor. The position of the F1' footprint is shown by a box and that of the footprint remaining after NF1 binding was eliminated by competition or mutation is denoted by a bracket labeled HX.
The essential element of the PBRU model then is that more than one sequence element within the 163-bp Sau3AI fragment is required to confer PB responsiveness. From our results we conclude that a central core of the fragment, between coordinates 22257 and 22207, is inactive alone but can confer PB responsiveness when combined either with upstream or downstream accessory elements within the fragment. In support of this hypothesis, one such accessory site, designated AF1, has been localized between coordinates 22188 and 22172. AF1 presumably represents an accessory factor binding site. The protein(s) responsible for the F0/F0 footprints may also be responsible for AF1 activity, since these footprints overlap with the AF1 sequence.

The level of PB responsiveness of construct 22257/22172 (Fig. 1B) is essentially the same as that of construct 22257/22155 (Fig. 1A), and both are about 2-fold lower than the wild-type 2317/2257 construct. This indicates that sequences between 22172 and 22155 are not essential for maximal PB responsiveness and, moreover, suggests that a second accessory element may be present between 2317 and 2257. Since full PB responsiveness is retained in 5' deletion constructs up to 2264, the putative second accessory element may lie between 2264 and 2257. However, it is also possible that the reduced responsiveness of the 2257/2155 construct (and of the 2257/2172 construct) is a consequence of partial inactivation of essential sequences at the 5' end of the central core. Experiments are currently underway to resolve these issues. In any case, removing one (or the other) of the putative CYP2B2 PBRU accessory sites reduces but does not abolish PB responsiveness. Similarly, eliminating any one of the three PEPCK GRU accessory sites reduces but does not abolish GRU-mediated glucocorticoid responsiveness (17).

Our results indicate that the HX-NF1 complex is required for maximal PB responsiveness (Fig. 6). NF1 proteins are abundant, ubiquitous transcription factors (37), different isoforms of which are products of a multigene family (38). The NF1 consensus binding sites are composed of two motifs, TGG and GCCAA, separated by a 6- or 7-bp spacer, and the protein protects a 25–30-bp region surrounding this sequence from DNase I digestion (25). An NF1 protein is clearly responsible for part of the F1/F1' footprint and the role of NF1 is positive, because mutations which reduce (e.g. NF1 m1) or abolish (e.g. NF1dm2) NF1 binding reduce but do not abolish PB responsiveness (Fig. 6). This result is similar to that of Honkakoski and Negishi (12) with a different NF1 mutation that reduced but did not eliminate PB responsiveness conferred by a subfragment of the mouse Cyp2b10 homolog of the CYP2B2 163-bp Sau3AI fragment. In our hands, mutations of the nuclear re-

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**Fig. 6.** Mutations of CYP2B2 PBRU sequence elements reduce PB responsiveness, but mutation of the Barbie box does not. Transfection experiments as well as analysis and presentation of CAT assay results were as described in the legend to Fig. 1. DNA sequences within the *shaded panel at left* represent the wild-type F1'-protected region, denoted WT, and various mutants of the HX-NF1 complex as shown. The mutated versions of the putative GRE and of the Barbie box are also shown at *left* as well as their sequence coordinates. For all mutant sequences, the upper strand is shown, and substitutions in the wild-type sequence are *underlined*. The F1'protected sequence is shown in double-stranded form at the *bottom*. *Brackets labeled HX and NF1 delimit the footprints attributed to proteins binding to the hexamer half-site and to the NF1 site, respectively.*

The mutated versions of the putative GRE and of the Barbie box are also shown at *left* as well as their sequence coordinates. For all mutant sequences, the upper strand is shown, and substitutions in the wild-type sequence are *underlined*. The F1'protected sequence is shown in double-stranded form at the *bottom*. *Brackets labeled HX and NF1 delimit the footprints attributed to proteins binding to the hexamer half-site and to the NF1 site, respectively.*
ceptor half-site element, as in HXM, or of both that element and NF1, as in HXm-NF1dm2, also reduce PB responsiveness, that is they have similar effects to those of mutational inactivation of NF1 alone (Fig. 6). In the mouse Cyp2b10 system, Honkakoski and Negishi (12) reported a more dramatic effect of such mutations in reducing PB responsiveness. Hence, the HX-NF1 complex is clearly essential for conferring maximal PB responsiveness.

The protein binding to the hexamer half-site creating the HX portion of the F1/F1′ footprint is unknown, but there are several possibilities. Inspection of the sequence surrounding the HX-NF1 complex reveals a recognition site for the orphan nuclear receptor, fetoprotein transcription factor (22, 39), and MatInspector finds a match to recognition sites for three other orphan receptors, including chicken ovalbumin upstream promoter transcription factor and HNF-4. Preliminary experiments have revealed binding of both HNF-4 (40) and fetoprotein transcription factor to the double-stranded HX oligo. Which of these or other proteins interacts functionally with the HX-NF1 complex to elicit maximal PB responsiveness remains to be determined.

The nature of the proteins responsible for the F0/F0′ and F2/F2′ footprints is currently unknown. They are of particular interest because F2/F2′ overlaps the corelet shared identified as essential for PB responsiveness whereas F0/F0′ overlaps the accessory AF1 site.

The protein responsible for the F3-F4/F3′ footprints is presumably a member of the nuclear receptor superfamily, because of the overlap with ER-7 site and because the F3-F4 footprint is competed by the HX oligo containing the AGGTCA hexamer half-site. The ER-7 region is not required for PB responsiveness, so it is clearly not an essential site, although it may be an accessory site. Similar conclusions were reached by Honkakoski and Negishi (12) with regard to the corresponding Cyp2b10 pB sequence.

Our observation that mutation of a putative GRE leads to a major reduction in PB responsiveness is particularly intriguing, as it raises the possibility that a glucocorticoid receptor-like molecule may be involved in conferring PB responsiveness. The putative PBRU GRE matches the GRE consensus at only 7 of 12 positions. Hence, like the GREs forming part of the PEPCK GRU (14), it is not a typical GRE.

Much previous effort has been concentrated on promoter-proximal sequences, particularly the Barbe box (29, 30) and their possible role in conferring PB responsiveness on the rat CYP2B1 and CYP2B2 genes. However, mutation of the Barbe box sequence in the context of the CYP2B2 promoter and 5′-flank does not affect PB responsiveness either in rat hepatocytes transfected by injection in situ (11) or in transfected primary rat hepatocytes (Fig. 6). Hence, while promoter-proximal sequences are doubtless required for basal transcriptional activity (9, 36), there is at present no convincing evidence for their involvement in conferring PB responsiveness.

The nucleotide sequences of the coding regions of CYP2B1 and CYP2B2 are about 97% identical (41, 42), and this extends over at least 1 kb of their 3′-flanking regions (43) and over some 2.3 kb of their 5′-flanking regions (13, 44). This, plus the fact that the responses of liver CYP2B1 and CYP2B2 to PB treatment are very similar (although the basal level of CYP2B2 is somewhat higher than that of CYP2B1 (45, 46), explains why CYP2B1 and CYP2B2 are sometimes treated as though they were a single gene (47, 48). Nevertheless, there are striking differences in the tissue-specific expression of CYP2B1 and CYP2B2, most notably in lung where CYP2B2 is not expressed, whereas CYP2B1 is expressed constitutively but is not PB-inducible (46). Because the 163-bp Sau3AI fragment is similarly positioned and, except for a single nucleotide difference, identical in sequence in CYP2B1 and CYP2B2 (13, 44) (see also Fig. 2), we will have to look elsewhere to explain the differences between CYP2B1 and CYP2B2 expression in the lung. It seems likely that the CYP2B1 PBRU and CYP2B2 PBRU are both inactive in the lung and that CYP2B1 expression is turned on because of the constitutive presence of some other transcription factor, the activity of which depends on one of the subtle sequence differences between the CYP2B1 and CYP2B2 5′-flanking regions.

The molecular details as to how the presence of PB leads to activation of transcription via the PBRU remain to be determined. However, a recent report of results obtained by in vivo footprinting suggests that PB may modify the interaction of transcription factors with the PBRU in chromatin (48). Although this provides further support for the role of the PBRU in transcriptional activation, further characterization of the proteins that bind to it will be necessary to elucidate the mechanism.

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REFERENCES

1. Guengerich, F. P. (ed) (1987) Mammalian Cytochromes P-450 Vol 1, CRC Press, Inc., Boca Raton, FL.
2. Guengerich, F. P., and Shimada, T. (1991) Chem. Res. Toxicol. 4, 391–407.
3. Gozani, O. J., and Shimada, T. (1990) Pharmacol. Ther. 45, 241–298.
4. Denison, M. S., and Whitlock, J. P., Jr. (1995) J. Biol. Chem. 270, 18175–18178.
5. Adensik, M., Bar-Nun, S., Masche, F., Zunich, M., Lippman, A., and Bard, E. (1981) J. Biol. Chem. 256, 10340–10345.
6. Hardwick, J. P., Gonzalez, F. J., and Kasper, C. B. (1983) J. Biol. Chem. 258, 8081–8085.
7. Waxman, D. J., and Azarof, L. (1992) Biochem. J. 281, 577–592.
8. Sinclair, P. R., Bement, W. J., Haugen, S. A., Sinclair, J. F., and Guzelian, P. S. (1999) Cancer Res. 59, 5219–5224.
9. Trottier, E., Belzil, A., Stoltz, C., and Anderson, A. (1999) Gene 158, 263–268.
10. Lee, P. Y. T., Adensik, M., Ganguly, S., and Shaw, P. M. (1996) Biochem. Pharmacol. 51, 345–356.
11. Park, Y., Li, H., and Kemper, B. (1996) J. Biol. Chem. 271, 23725–23728.
12. Honkakoski, P., and Negishi, M. (1997) J. Biol. Chem. 272, 14943–14949.
13. Hoffmann, M., Mager, W. H., Scholte, B. J., Civil, A., and Planta, R. J. (1992) Gene Expr. 2, 353–363.
14. Isel, E., Stromstedt, P.-E., Quinn, P. G., Carlstedt-Duke, J., Gustafsson, J.-Å., and Granner, D. K. (1996) Mol. Cell. Biol. 10, 4712–4719.
15. Hall, R. K., Sladek, F. M., and Granner, D. K. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 412–416.
16. Wang, J.-C., Stromstedt, P.-E., O’Brien, R. M., and Granner, D. K. (1996) Mol. Endocrinol. 10, 794–800.
17. Scott, D. K., Mitchell, A., and Granner, D. K. (1996) J. Biol. Chem. 271, 3109–3114.
18. Scott, D. K., Mitchell, A., and Granner, D. K. (1996) J. Biol. Chem. 271, 3109–3114.
19. Jacoby, D. B., Zink, N. D., and ToweI, H. C. (1989) J. Biol. Chem. 264, 17623–17626.
20. Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982) Mol. Cell. Biol. 2, 1044–1051.
21. Seed, B., and Shen, J.-Y. (1988) Gene (Amst.) 67, 271–277.
22. Bernier, D., Thumasson, H., Allard, D., Guertin, M., Hamel, D., Blaquiere, M., Berachemin, M., LaRue, H., Estable-Puig, M., and Belanger, L. (1983) Mol. Cell. Biol. 13, 1619–1633.
23. Labbé, D., Jean, A., and Anderson, A. (1988) DNA (N.Y.) 7, 253–260.
24. Quandt, K., Frech, K., Karas, H., Wingender, E., and Werner, T. (1995) Nucleic Acids Res. 23, 4878–4884.
25. Gronostajski, R. M., Ahya, S., Nagakura, K., Gugenheim, R. A., and Hurwitz, J. (1985) Mol. Cell. Biol. 5, 964–971.
26. Tsai, M.-J., and O’Malley, B. W. (1994) Annu. Rev. Biochem. 63, 451–486.
27. Jiang, G. Q., Nepomuceno, L., Hopkins, K., and Sladek, F. M. (1995) Mol. Cell. Biol. 15, 5131–5143.
28. Beato, M. (1989) Cell 56, 325–344.
29. Liang, W., He, J.-S., and Fuchs, A. J. (1995) J. Biol. Chem. 270, 4438–4450.
30. He, J.-S., and Fuchs, A. J. (1995) J. Biol. Chem. 266, 7864–7869.
31. Grosveld, F., van Assendelft, D. G., Greaves, D. R., and Kollis, G. (1987) Cell 51, 975–985.
32. Ramsden, R., Sommer, K. M., and Omiecinski, C. J. (1993) J. Biol. Chem. 268, 21722–21726
33. Schüle, R., Muller, M., Kaltschmidt, C., and Renkawitz, R. (1988) Science 242, 1418–1320
34. Woodward, R. N., Li, M., and Holland, L. J. (1997) Mol. Endocrinol. 11, 563–576
35. Anderson, A., Trottier, E., Stoltz, C., Vachon, M.-H., Paquet, Y., and Dubois, S. (1997) FASEB J. 11, 777
36. Honkakoski, P., and Negishi, M. (1998) J. Biochem. Mol. Toxicol. 12, 3–9
37. Faisst, S., and Meyer, S. (1992) Nucleic Acids Res. 20, 3–26
38. Rupp, R. A. W., Kruse, U., Malthaup, G., Göbel, U., Beyreuther, K., and Sippel, A. E. (1990) Nucleic Acids Res. 18, 2607–2616
39. Galarneau, L., Paré, J.-F., Allard, D., Hamel, D., Lévesque, L., Tugwood, J., Green, S., and Belanger, L. (1996) Mol. Cell. Biol. 16, 3853–3865
40. Trottier, É. (1997) Étude de la Sous-famille 2B de Cytochromes P450 du Rat: Characterisation de Nouveaux Génés et Régulation de CYP2B2 par le Phenobarbital. Ph.D. thesis, Université Laval
41. Suwa, Y., Mizukami, Y., Sogawa, K., and Fujii-Kuriyama, Y. (1985) J. Biol. Chem. 260, 7980–7984
42. Mizukami, Y., Sogawa, K., Suwa, Y., Muramatsu, M., and Fujii-Kuriyama, Y. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 3958–3962
43. Jean, A., Rivkin, E., and Anderson, A. (1988) DNA (N. Y.) 7, 361–369
44. Shaw, P. M., Edigkaufier, M., Doehmer, J., and Adesnik, M. (1996) Biochim. Biophys. Acta 1305, 54–58
45. Christou, M., Wilson, N. M., and Jefcoate, C. R. (1987) Arch. Biochem. Biophys. 258, 519–534
46. Wilson, N. M., Christou, M., and Jefcoate, C. R. (1987) Arch. Biochem. Biophys. 256, 407–420
47. Prabhu, L., Upadhaya, P., Ram, N., Nirodi, C. S., Sultana, S., Vatsala, P. G., Mani, S. A., Rangarajan, P. N., Surolia, A., and Padmanaban, G. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9628–9632
48. Kim, J., and Kemper, B. (1997) J. Biol. Chem. 272, 29423–29425
49. Trottier, E., Dubois, S., Vachon, M.-H., Stoltz, C., Belzil, A., and Anderson, A. (1996) Xth International Symposium on Microsomes and Drug Oxidations, Los Angeles, July 21–24, 1996, Abstract P-149, Los Angeles Organizing Committee, Los Angeles, CA
The CYP2B2 Phenobarbital Response Unit Contains an Accessory Factor Element and a Putative Glucocorticoid Response Element Essential for Conferring Maximal Phenobarbital Responsiveness

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