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IDENTIFICATION AND CHARACTERIZATION OF ELEMENTS REGULATING THE EXPRESSION OF THE PHENOBARBITAL-INDUCIBLE CYP2B1 AND CYP2B2 GENES

by

Karen Marie Sommer

A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

University of Washington

1996

Approved by 

Chairperson of Supervisory Committee

Program Authorized to Offer Degree ENVIRONMENTAL HEALTH

Date AUGUST 15, 1996

Doctoral Dissertation

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Abstract

IDENTIFICATION AND CHARACTERIZATION OF ELEMENTS REGULATING THE EXPRESSION OF THE PHENOBARBITAL-INDUCIBLE CYP2B1 AND CYP2B2 GENES

by Karen Marie Sommer

Chairperson of the Supervisory Committee: Curtis Omiecinski, PhD
Department of Environmental Health

Phenobarbital (PB) is the prototype for a class of agents that produce marked induction in the genetic expression of a variety of metabolic enzymes in many species. One of the consequences of this induction is a change in the metabolic fate and disposition of xenobiotics, which in turn influences their pharmacologic efficacy or toxicity. The biological mechanisms effecting these induction processes are not understood. The cytochromes P450 (CYP) 2B1 and CYP2B2 are the major PB-inducible genes in the rat, and have been the most intensively studied. Although PB-induction of these genes occurs via an increase in gene transcription initiation, cis-acting DNA sequences involved have not been identified. This is largely due to the difficulty of maintaining PB-inducibility in cultured cells. The aim of this dissertation research is to identify and characterize cis-acting elements involved in regulating CYP2B1/2B2 expression.

In the initial phase of this research transgenic mice carrying rat CYP2B2 gene constructs were analyzed. Transgenes lacking the section of the CYP2B2 gene between -20 and -.8kb were expressed at high levels regardless of PB exposure. This suggests a cis-acting element 5' of -.8kb represses CYP2B2 transcription, which is reversed in the presence of PB. Transient transfection studies also provided evidence of repressor elements regulating CYP2B1/2B2 genes. Successive 5' deletions of their flanking sequences increased CAT reporter gene expression, indicating that multiple repressor elements reside in the 5' sequence. A number of *in vitro* methods were used to analyze protein-DNA interactions occuring along the CYP2B1/2B2 5' sequence. Proteins binding a recognition site at -2.2kb were further characterized according to their binding site affinity and immunoreactivity to

antibodies raised against known transcription factors and specific phospho-amino acids. This element was identified as a recognition motif for the transcription factor NF-1. The NF-1 binding site is present in CYP2B2 DNA identified by others as required for PB-inducibility, suggesting an important role for this protein in the PB induction response. NF-1 binds in both control and PB-induced states, but has a higher affinity after PB treatment. Increased binding is not correlated with NF1-L transcript levels nor differential reactivity to anti-phospho-amino acid antibodies.

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LIST OF ABBREVIATIONS

BTE basal transcription element

CAT chloramphenicol acetyltransferase

CYP cytochrome P450

EMSA electrophoretic mobility shift assay

GRE glucocorticoid response element

HNF hepatic nuclear factor

NF-1 nuclear factor 1

PB phenobarbital

PMA phorbol 12-myristate 13-acetate

RSV Rous sarcoma virus

TCDD 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin

TGF-β transforming growth factor-β

ACKNOWLEDGMENTS

I would like to express my gratitude to my advisor, Dr. Curt Omiecinski. I am very thankful for the opportunity to work on this exciting research project, and am particularly grateful for his guidance, support, and encouragement along the way. I would also like to thank two co-workers in the Omiecinski laboratory, Richard Ramsden and Dr. Chris Hassett, without whom I would not have been able to complete this research. Their technical expertise, generosity, and sense of humor have been of enormous assistance to me! I am also indebted to Dr. Jaspreet Sidhu and Nancy Beck for their contributions to this project.

There are many others in the department who have been instrumental in various aspects of this research project: Mara Seeley, Sandy Kirchner, Rafael Ponce, Diane Neil, Colin White, Dennis Sloan, Fred Farin, Mark Anderson, Sarah Brainard, Kirk Van Ness, Jesara Schroeder, Sally Thompson, Joe Walker, Dr. David Eaton, Dr. Terry Kavanagh, Dr. James Woods, Dr. Elaine Faustman, Azure Skye, Laurie Aicher, Kirsten Robinson, Susan Silbernagel, as well as all of my co-workers in the Omiecinski lab.

I am very grateful to the members of my dissertation committee: Dr. Terry Kavanagh, Dr. Curt Omiecinski, Dr. Richard Palmiter, Dr. Allan Rettie, and Dr. George Todaro. Their valuable input in this dissertation research was highly appreciated, and I am very honored to have them all on my committee.

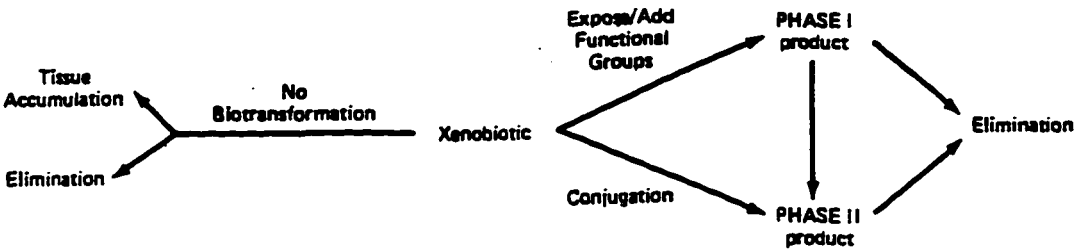
Finally, I would like express my deepest gratitude to my parents, Harmon and Maxine Sommer, for all of their support, encouragement, love, and prayers throughout the years. I am also profoundly thankful to my sisters, Kathi, Kirsten, and Kristine, and to my brother and his family, Doug, Suzanne, Daniel, and Grant Sommer, for all of their love and support.

INTRODUCTION

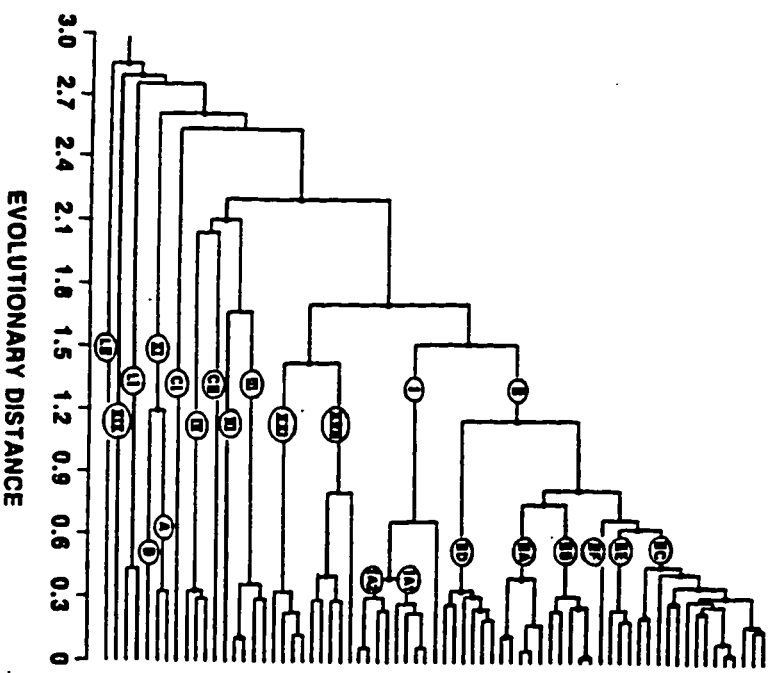
THE CYTOCHROME P450 SUPERFAMILY

Enzymes involved in xenobiotic metabolism can be divided into two classes: phase I enzymes, which catalyze oxidative reactions to a diverse array of chemical substrates; and phase II enzymes whose primary role is the transfer of large hydrophilic moieties to functional groups introduced by phase I enzymes or inherent in the xenobiotic (see next page). The net result is generally detoxification of the substrate, via increasing its hydrophilic character and thus its somatic clearance. For some chemicals, however, phase I oxidation can generate metabolites which are more reactive with biological molecules than the parent compound, called bioactivation. Alterations in levels of both phase I and II enzymes can greatly affect xenobiotic disposition, influencing chemical toxicity or pharmacologic properties.

The majority of enzymes catalyzing phase I metabolism are members of the cytochrome P450 superfamily (CYP). These heme-containing microsomal enzymes are prevalent in the liver and present in virtually every tissue. The second figure on the next page shows a phylogenetic tree diagramming the evolutionary relationship between CYP family members. CYP members of importance to xenobiotic metabolism (I and II in this diagram; CYP1 and CYP2 in current nomenclature) are thought to have diverged relatively recently from genes encoding CYPs that metabolized endogenous chemicals such as steroids and fatty acids, e.g. LII, XI, XIX (1). The notable expansion of the CYP1 and CYP2 subfamilies within the last 400 million years is postulated to have arisen in response to changes in fauna and dietary habits (2). The result of these many gene conversion events is a battery of enzymes, each having multiple substrate specificities, with the ability to metabolize xenobiotics with a diverse array of chemical structures.



Integration of phase I and phase II biotransformation reactions (3).



P450 protein phylogenetic tree. The divergence times were calculated as described by Nelson and Strobel (1). A total of 69 P450 sequences were compared (2).

INDUCTION OF CYTOCHROME P450 ENZYMES BY XENOBIOTICS

MECHANISMS OF INDUCTION

The activity of some CYP enzymes can be induced with exposure to certain compounds. This was described in early studies on the basis of the alterations this produces in drug pharmacologic response, such as a decrease in sleep time in rats exposed to the sedative PB after chronic exposure (4). At the molecular level, the most well-known instance of CYP induction is the increase in CYP1A1 gene transcription upon exposure to aromatic hydrocarbons (such as TCDD and 3-methylcholanthrene) via the Ah receptor. This mechanism, whereby a xenobiotic acts as the ligand activating a transcription factor that can upregulate CYP genes involved in its own metabolism, is not generally found among inducible CYP genes. The following table lists some of the mechanisms governing induction of other CYP genes by xenobiotics.

Table of CYP drug induction mechanisms (5).

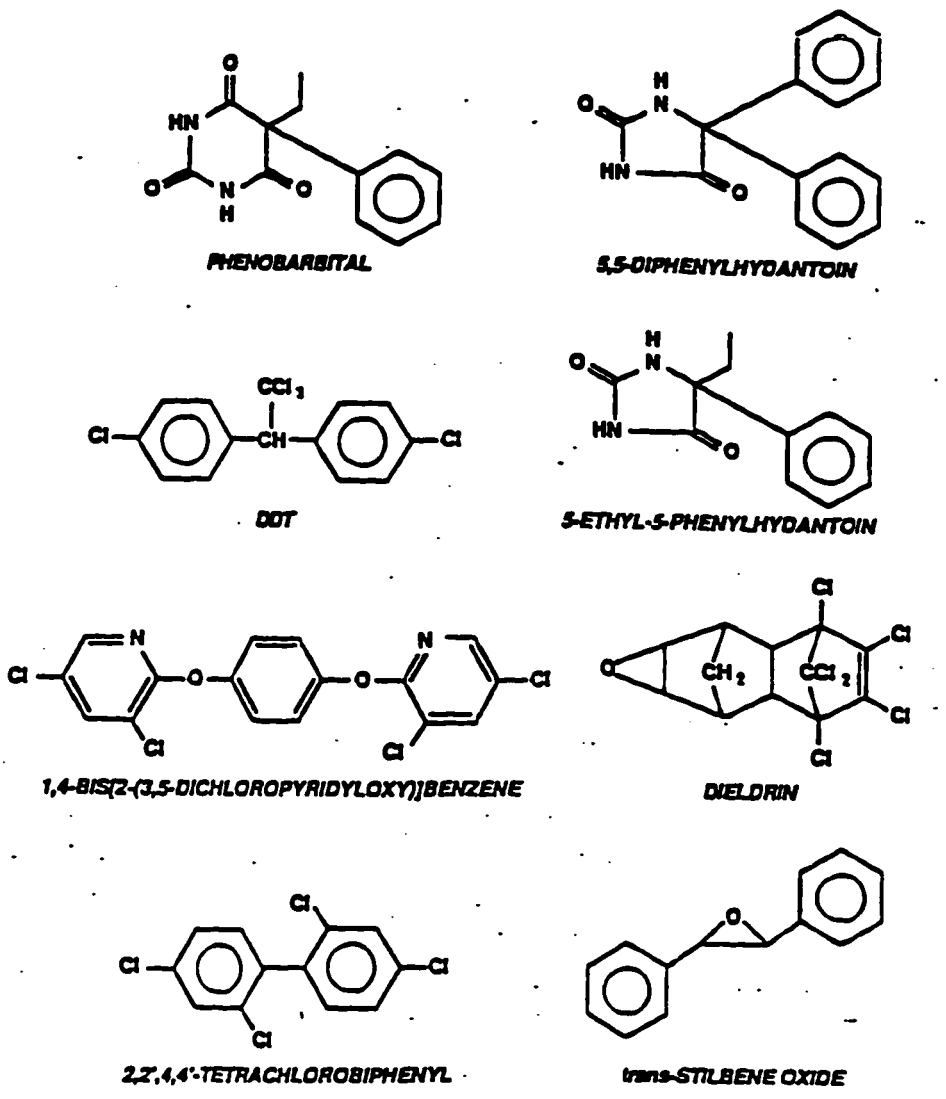
CYP form	prototypic inducers	primary induction mechanism
1A1	dioxin, 3-methylcholanthrene	transcription activation by ligand-activated Ah receptor
1A2	3-methylcholanthrene	mRNA stabilization
2B1, 2B2	phenobarbital	transcriptional activation
2E1	ethanol, acetone, isoniazid	protein stabilization
3A1	dexamethasone	transcriptional activation; independent from classical glucocorticoid receptor pathway
4A1	clofibrate	transcriptional activation by peroxisome-proliferator-activated receptor

PHENOBARBITAL-LIKE INDUCERS

PB is actually the prototype for a class of chemicals, termed PB-like inducers, that can activate transcription of a number of genes encoding xenobiotic metabolizing enzymes, including CYP2B1, CYP2B2, CYP3A1, aldehyde dehydrogenase, NADPH-P450-oxidoreductase, and the phase II enzymes glutathione-S-transferase and UDP-glucuronyl transferase. As the previous table indicates, the mechanism by which PB induces transcription is unknown. Several experiments suggest it is unlikely a ligand-activated transcription factor comparable to the Ah receptor is involved. Firstly, there is no evidence of any cellular protein with affinity for PB-like inducers (6). Secondly there is also no apparent structure-activity relationship between PB-like inducers and transcriptional activation (5,7). Instead, PB-like inducers consist of a wide variety of chemical structures as illustrated on the next page. Thirdly, the two enantiomers of the PB-like inducer 5-ethyl-5-phenylhydantoin both activate transcription as effectively as the racemic mixture (8).

Although the mechanism is obscure, certain facts are known about the PB induction response which have a direct bearing on the experiments to be presented here. The most important of these is that PB induces CYP2B1 and CYP2B2 expression through an increase in transcription initiation events. Nuclear run-on assays can detect an increase in transcription within 30 minutes of PB exposure (9). Maximum transcription activation occurs 4 hours after PB exposure, plateaus, then gradually diminishes over 24 hours (10). Levels of the CYP2B1 and CYP2B2 RNAs increase during this time course with peak levels about 16-18 hours after PB induction (10). This induction at the level of gene transcription appears to require protein synthesis. Pre-treatment of rats with cycloheximide blocks both PB induced increases in CYP2B1 and CYP2B2 transcription rates as well as mRNA accumulation (11).

The PB-induction response is highly differentiation- and tissue-specific. In the rat, PB induction of CYP2B1 and CYP2B2 genes occurs extensively in the liver, only slightly in adrenal and small intestine tissues, and not in lung and testes (5). Hepatic CYP2B1 and CYP2B2 genes in the developing rat fetus can be PB-induced beginning at gestation day 22 (12). Another marker for hepatic differentiation, HNF-1, first appears at gestation day 15 (13). The requirement for a highly-differentiated hepatic phenotype has made it difficult to



Structures of some PB-type inducers (7)

find a cell culture system to study PB induction. Established hepatoma cell lines are unable to express CYP2B1 or CYP2B2 either constitutively or with PB treatment. Primary hepatocytes undergo de-differentiation in culture, losing the ability to respond to PB. Further understanding of the roles of extracellular matrix and culture medium in modulating cellular phenotypes led to significant advances in this area. Primary hepatocyte cell culture systems that are able to maintain liver-specific morphology and functions, including CYP2B1 and CYP2B2 expression in response to PB, have been described (14,15) These are suitable for analyzing the cell biology of PB induction, but are as yet refractory to transfection with exogenous DNA.

Recent investigations have begun to define the role of phosphorylation events in mediating the PB response. In PB-inducible primary rat hepatocytes, incubation with medium containing cAMP analogs in addition to PB eliminates the CYP2B1 and CYP2B2 induction observed with PB alone (16). In similar experiments, cells were exposed to specific inhibitors of different phoshorylation and de-phosphorylation pathways along with PB. PB-induced CYP2B1 and CYP2B2 levels were down-regulated in cells incubated with okadaic acid (17), an inhibitor of the nuclear serine/threonine protein phosphatases PP2A, PP1 and PP3 (18).

CYP2B1 AND CYP2B2

EXPRESSION

CYP2B1 and CYP2B2 are the major PB-inducible genes in the rat, and as such their inducible, developmental, and tissue-specific expression patterns are well defined. They share a 97% sequence identity that extends throughout the coding sequence and includes the first 2.5kb of 5' DNA (19). Considering this similarity in nucleotide structure, CYP2B1 and CYP2B2 expression patterns are suprisingly distinct. CYP2B2 but not CYP2B1 is expressed constitutively at low levels in liver. However, after exposure to PB, CYP2B1 transcript levels are present at approximately 5-fold higher levels than CYP2B2 (20). Conversely, CYP2B1 but not CYP2B2 shows constitutive expression in the lung and testes (20).

GENE REGULATION

Because of the difficulty in transfecting PB-inducible primary hepatocytes, functional studies of cis-acting sequences regulating CYP2B1 and CYP2B2 expression are lacking. Most of what is known about the mechanism of their induction has been gained using hepatic nuclear extracts of PB-treated rats. At the time this project was begun, other investigators had just identified a 17bp element in CYP2B1 and CYP2B2 between -89 to -73 that bound nuclear protein from PB-induced rats greater than those from uninduced in electrophoretic mobility shift assays (EMSAs). This element was initially identified as a highly conserved nucleotide sequence between the upstream control region of the PB-inducible *Bacillus megatereum* $CYP_{bm\text{-}3}$ gene and the 5' region of the CYP2B1 and CYP2B2 genes (21). This element will be referred to as 17b/e in this paper. Two *in vitro* transcription assays of CYP2B2 promoter deletion mutants reported a positive cis-acting element just 5' of -75bp (19,22). In these assays the CYP2B2 promoter can direct significant transcriptional activation in control proteins which is either increased 8 fold (22) or only slightly (19) with PB protein. On the basis of these studies the 17b/e element has been termed the "barbie box", for its putative role in phenobarbital induction.

CHAPTER 1: PHENOBARBITAL INDUCTION AND TISSUE-SPECIFIC EXPRESSION OF THE RAT CYP2B2 GENE IN TRANSGENIC MICE

SUMMARY

Transgenic mouse lines were developed that carried the 19kb CYP2B2 gene and either 0.8 or 20kbp of 5' flanking DNA. The PB induction response required the presence of sequences 5' of -0.8kb. In the absence of these sequences, constitutive transgene expression occurs at high levels, and was not induced with PB treatment.

INTRODUCTION

There were two specific aims for the experiments that will be presented in this chapter. The first was to test the suitability of transgenic mice as a PB-inducible system for studying cis-acting CYP2B sequences. Induction of CYP family members by PB-like compounds is known to occur across a number of species including humans (23), mice (24), chickens (25), and bacteria (26). In the mouse, PB increases the expression of orthologues of the rat CYP2B1 and CYP2B2 genes, Cyp2b-9 and Cyp2b-10 (27,28). If proteins regulating PB induced gene expression are also conserved between these two species, transgenic mouse experiments could provide much-needed functional information on cis-acting rat CYP2B1 or CYP2B2 sequences in a PB-inducible cell system.

When this project was begun, little information on cis-acting CYP2B2 genetic elements was available. Therefore, the second specific aim of this project was to broadly define sections of the CYP2B2 gene that are involved in PB induced transcription activation. Genetic regulatory elements are commonly located within 5' gene flanking DNA; however, they can also be found in exonic (29,30), intronic (31), and 3' sequence (32). Richard Ramsden in the Omiecinski laboratory had created two CYP2B2 gene constructs for use in transgenic mice. Both contained the entire CYP2B2 gene including all exons, introns, and 4kb of 3' DNA. They differed only in the amount of 5' flanking sequence included: either 20kb or 0.8kb. If PB-inducible expression was found in only one construct, the amount of

DNA in which to search for cis-acting elements required for PB induction would be cut in half.

These two constructs would also allow us to further evaluate the regulatory contribution of proximal elements, implicated in the PB response by other investigators, and more distal protein binding loci identified in our laboratory at approximately -1400 and -2200bp (see Chapter 3). The main proximal element of interest, 17b/e, had been identified by Fulco and colleagues (21,26,33) and implicated as mediating barbiturate-regulated protein binding and the corresponding transcriptional activation response. This 17-bp element maps to a position at -73bp upstream from the transcription start site in the CYP2B1 and CYP2B2 genes and was reported to interact avidly with a nuclear protein from PB-treated liver (21). In separate studies, Padmandaban and co-workers (34) proposed that this site, which in footprinting experiments mapped to -56 to -88 of the CYP2B2 gene, was critical in mediating PB transcriptional activation. This element would be isolated from the upstream protein binding sites at -1400 and -2200bp in constructs with only 802bp of 5' sequence. All experiments reported in this chapter were performed with the equal participation of Richard Ramsden in the Omiecinski laboratory

RESULTS

Analysis of Transgenic Expression

Four mouse strains were founded, two of which contained the rat CYP2B2 transgenes with 20kb, and two which contained only the proximal 802bp, of 5'-genetic sequence (Figure 1). Analyses of Southern blot phosphorimaging data indicated that mouse strain 39E-B possessed a single copy of the transgene while strain 39E-Y maintained about 3 copies (Table I). When RNA derived from male mice from either of these strains was probed with an oligonucleotide specific for the CYP2B2 transgene, only RNA from PB-induced mouse liver exhibited high level expression of CYP2B2 (Figure 2, panel B). Upon longer exposures of the same blot, a very low level of CYP2B2 mRNA also was detectable in livers from uninduced transgenic mice carrying the long CYP2B2 construct (data not shown). To assess general PB responsiveness in mice, Northern blot hybridizations were performed with an oligonucleotide specific for the endogenous mouse

PB-inducible P450, Cyp2b9. The results obtained from these hybridizations confirmed PB inducibility of the mice from both transgenic and nontransgenic lines and revealed the constitutive expression of CYP2b9 in the lung of all animals (Figure 2, panels A and B). In female mouse liver, assessed independently, relatively high constitutive levels of Cyp2b9 were detected. However, this sex difference noted for basal expression levels of the endogenous mouse CYP2b9 gene was not observed for the CYP2B2 transgene, which exhibited low level expression in both sexes of the 39E lines. Therefore, in 39E mice, which contain the long CYP2B2 construct that includes 20kb of 5'-flanking sequence, PB induction and tissue-selective expression profiles of the transgene were retained as characterized previously for the endogenous CYP2B2 gene in the rat (20).

Table I. Estimated copy number and relative RNA expression levels of CYP2B2 in transgenic mouse lines

		Percent CYP2B2 expression relative to adult rat PB-induced liver					
		kidney			liver		
Transgene constrct/colony	copy number	C	PB	change	C	PB	change
19E-O	20	378 (n=5)	354 (n=3)	-6%	300 (n=9)	126 (n=7)	-238%
19E-R	8	292 (n=3)	226 (n=3)	-23%	109 (n=3)	56 (n=3)	-195%
39E-B	1	ND (n=4)	ND (n=4)	ND	6 (n=4)	49 (n=6)	816%
39E-Y	3	ND (n=4)	ND (n=4)	ND	ND (n=4)	56 (n=5)	+++
NT-O, R, B, Y	0	ND (n=4)	ND (n=4)	ND	ND (n=4)	ND (n=4)	ND

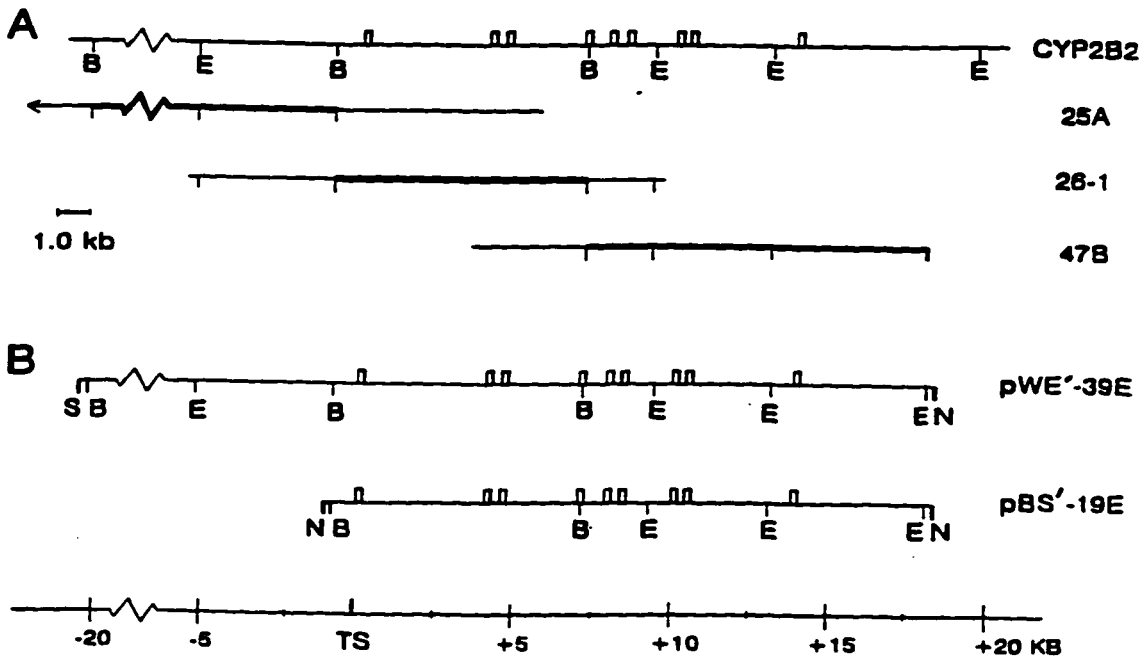


Figure 1. CYP2B2 genes in transgenic mouse experiments

Panel A schematizes the full-length CYP2B2 gene, indicating the portions (boldface regions) of three individual clones, 25A, 26-1, and 47B, which were excised and ligated to form the full-length gene construct. *Panel B* depicts the two full-length clone constructs which were used to generate the transgenic mouse lines in the current study. The cosmid, pWE'-39E, contained a 39-kb insert comprising the CYP2B2 gene, including 20kb of 5' flanking sequence. The insert from cosmid pWE'-39E was used to generate the 39E mouse lines. The plasmid, pBS'-19E, was identical across the CYP2B2 gene sequence as pWE'-39E, except that the former contained only 0.8kb of 5'-flanking sequence. The pBS'-19E insert was used to generate the 19E mouse lines. *B=Bam*HI; *E=Eco*RI; *N=Not*I; *S=Sfi*I; *TS*=transcription start site. *Open boxes* indicate positions of exons 1-9.

Two mouse colonies also were founded which contained a truncated transgene with 0.8kb of 5'-flanking sequence; colony 19E-O contained about 20 copies, and colony 19E-R had approximately 8 copies (Table I). Hybridization studies indicated that these animals expressed high levels of RNA for CYP2B2 in kidney and liver (Figure 2, panel A), but in a non-PB-dependent manner, *i.e.* CYP2B2 RNA was detected in both induced and uninduced mice. Hybridization assays with the CYP2b9 control probe confirmed the PB responsiveness of transgenic mice possessing the 0.8kb 5'constructs.

Although data presented in Figure 2 depicts only two of the four transgenic groups, both 39E colonies, and both 19E colonies, had patterns and levels of transgene expression that were analogous for each of the respective constructs. When comparing hepatic mRNA expression levels across all transgenic groups, the PB-induced 39E-B and 39E-Y animals expressed CYP2B2 at approximately 50% of the level of adult PB-treated rat liver (Table I) whereas the constitutive CYP2B2 hepatic levels of 19E-O (~20 copies) and 19E-R (~8 copies) animals were 3- and 1- fold that of the PB induced adult rat, respectively (Table I). These results indicated that CYP2B2 expression levels were roughly proportional to copy number in the liver. It was noteworthy that CYP2B2 transgene expression levels in livers of both 19E groups were consistently decreased as a consequence of PB treatment relative to approximately 50% of the levels measured in uninduced animals.

CYP2B2 Protein Analysis in Transgenic Mice

To affirm the expression of the transgenes at the protein level, Western blot analyses of microsomal proteins from 39E-B and 19E-O transgenic livers were conducted using a CYP2B1/2B2 polyclonal antibody. The results presented in Figure 3 demonstrate an immunologically distinct band migrates at the predicted size for CYP2B2 in the transgenic animals. In addition, the endogenous mouse Cyp2b9 protein is detected on these blots in both transgenic and non-transgenic mice, migrating at a slightly higher molecular mass. In the 19E-O transgenic kidney, CYP2B2 protein also is detected; immunocytochemical

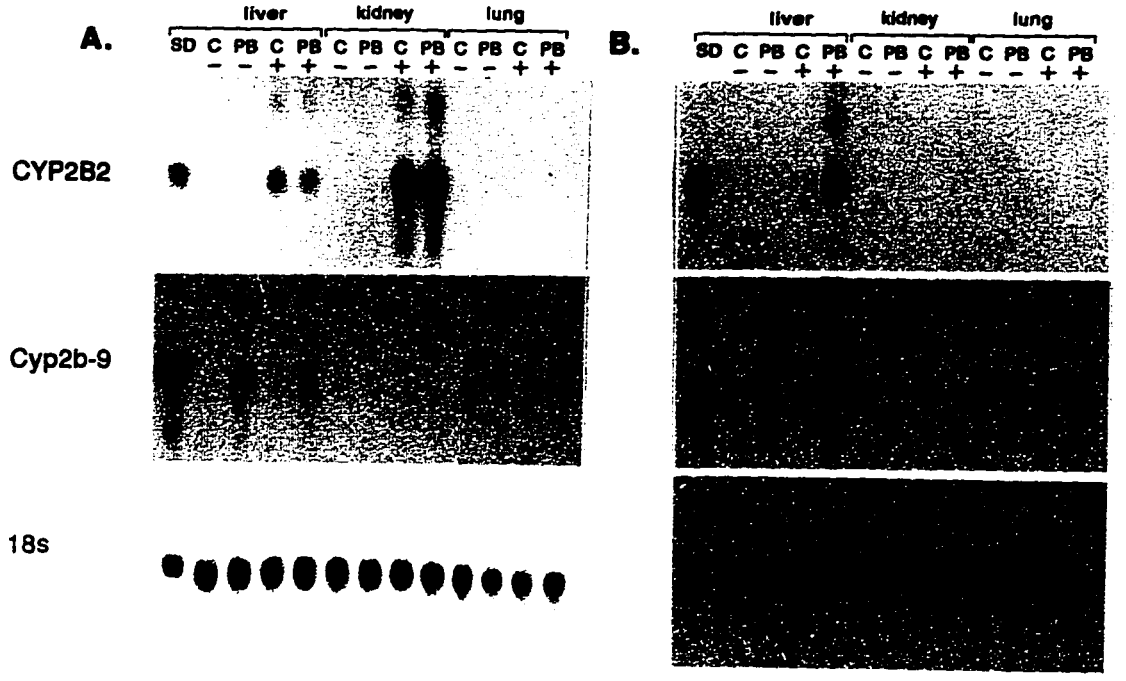


Figure 2. Northern blot analyses of RNA expression profiles in transgenic mice

Ten µg of total RNA was loaded on each lane except for the SD (Sprague-Dawley PB-induced rat liver) lanes which contain 5µg of RNA. The blots were probed with an oligomer specific for CYP2B2 *(upper panel)*, mouse CYP2b9 *(middle panel)*, and 18S ribosomal RNA to evaluate relative loading efficiency of RNA samples *(lower panel)*. *Panel A*, RNA hybridization data obtained from male 19E-O mouse tissues. *Panel B*, RNA hybridization data obtained with male 39-E-B mouse tissues. The designations "*C*" and "*PB*" refer to control and phenobarbital pretreatments, respectively. The "-" and "+" designations refer to nontransgenic littermates and transgenic mice, respectively.

studies demonstrated that the reactive protein in this organ was restricted mainly to the proximal tubules.

Tissue-Specific Expression of the CYP2B2 Transgene

Results of an RNA slot blot hybridization study are presented in Figure 4 that illustrate tissue-specific expression profiles of the short (-0.8kb 5') and long (20kb 5') CYP2B2 transgenes in mice, together with the endogenous Cyp2b9 mouse gene. In total, 10 tissues were surveyed in both untreated and PB-treated, male and female mice, including the brain, testes, ovary, heart, thymus, spleen, salivary gland, kidney, lung, and liver. Figure 4 presents representative data obtained form male mice, from two of the transgenic mouse lines examined. The Cyp2b9 gene was detected primarily in PB-treated liver, but low levels were also apparent in lung. Although very low levels of expression of the -0.8kb CYP2B2 transgene appeared to be detectable in testes, no evidence for its expression occured in any other tissues except for liver and kidney as described previously. Detection of the longer -20kb transgene appeared to be limited to the liver with these assay procedures. It was of interest to note that expression of the rat CYP2B2 gene constructs, even at relatively high levels, appeared to confer no deleterious effects on any of the transgenic animals, nor any impairment in their reproductive success.

DISCUSSION

To assess, in vivo, the contributions of 5'-flanking proximal and remote gene sequences in the PB induction response, expression patterns in mice transgenic for full-length rat CYP2B2 transcriptional units that differed only in their respective 5'-upstream sequences were examined. Mice maintining 19-kb CYP2B2 genes, including 0.8kb of 5'-flanking gene sequence and complete with all introns, coding exons, and 4kb of 3'-flanking sequence, were *not* activated by PB either at the level of mRNA or protein expression.

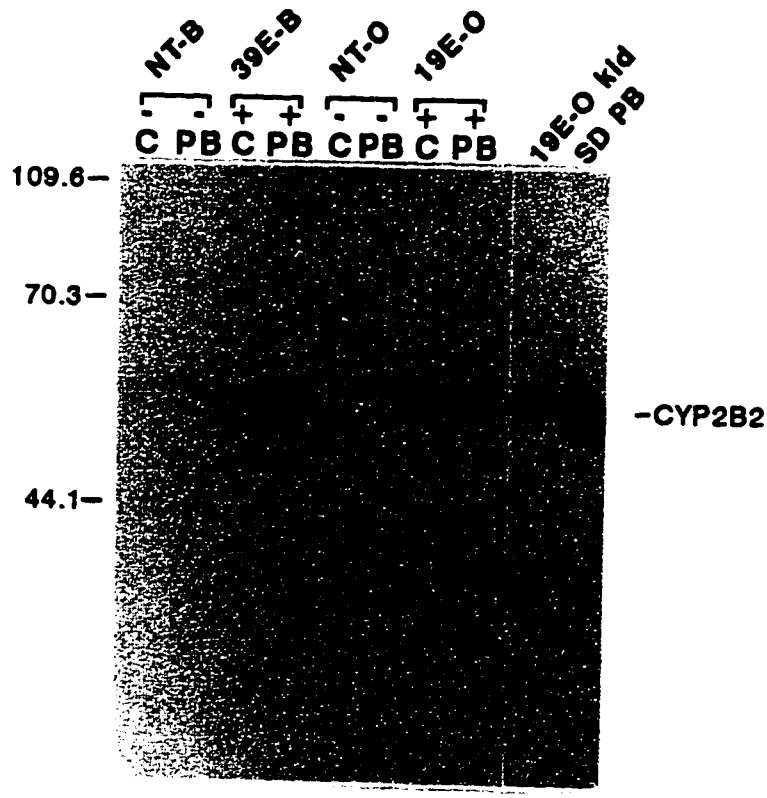


Figure 3. Western blot analysis of hepatic microsomal proteins in transgenic and nontransgenic mice.

One μg of hepatic microsomal protein was loaded on each lane of the polyacrylamide gel, electrophoresed, transferred to an Immobilon-P membrane (Millipore), and reacted with an antibody specific for CYP2B (see "Materials and Methods"). Data obtained from the 19E-O and 39E-B lines are presented. The designations, *C* and *PB*, refer to control and phenobarbital pretreatments, respectively. The "-" and "+" designations refer to nontransgenic littermates and transgenic mice, respectively. *NT*, nontransgenic littermates. The "39E-B" line possesses one copy of the 39-kb CYP2B2 gene, and the "19E-O" line possesses 20 copies of the 19-kb CYP2B2 construct. For comparison, a kidney microsomal protein lane is included *(19E-O kid)*, as well as a microsomal protein sample obtained from Sprague-Dawley PB-induced rat liver *(SD-PB)*.

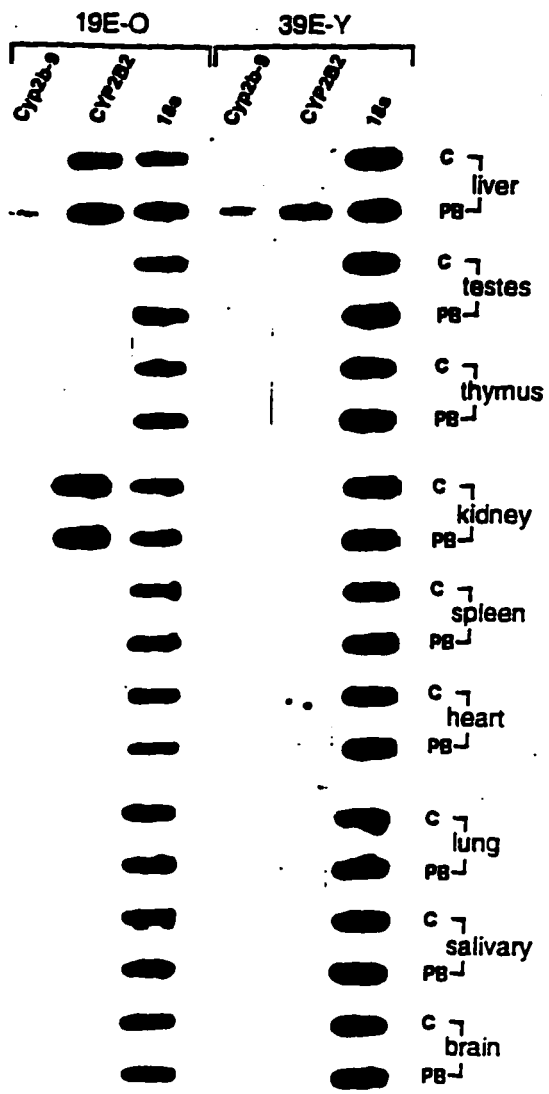


Figure 4. RNA slot blot analysis of tissue-specific expression of the CYP2B2 transgene and the mouse Cyp2b9 in control and PB-induced transgenic mouse lines.

RNA was prepared from each tissue as described under "Materials and Methods". Five μg of total RNA extracts were loaded in each lane. The membranes were hybridized to either the CYP2B2 or Cyp2b9-specific probes and subjected to autoradiography. A ribosomal 18S hybridization probe was used to assess loading equivalency across the membrane.

In contrast to the expression patterns noted for the endogenous rat CYP2B2 gene, structural products of the -0.8kb constructs were expressed at very high constitutive levels in transgenic mouse kidney and liver. PB administration to these mice actually resulted in down-regulation of hepatic transgene expression (Figure 2; Table I). In contrast, mice maintaining similar CYP2B2 transgenes, but with an additional 19kb of 5' flanking sequence, were positively regulated by PB treatments and exhibited liver-specific profiles highly analogous to the endogenous CYP2B2 gene expression profiles in the rat (20,35,36). Mice with 39-kb transgenes, containing 20kb of 5'-flanking sequence, exhibited no observed constitutive expression of CYP2B2 in any tissue, except for very low levels in the liver. Therefore, with respect to both PB induction and tissue-specific expression, the 39-kb CYP2B2 construct, but not the 19-kb construct, was regulated in the mouse apparently identically to the parental gene of the rat. We conclude from these observations that the presence of sequences within -0.8kb to -20kb of the 5'-flanking region of the rat CYP2B2 gene confer critical regulatory information necessary for PB induction and tissue-specific expression *in vivo*.

Previous reports have indicated that a 17- or 32-bp genetic element between -50 and -90 relative to the transcription initiation site of CYP2B2 interacts with PB-modulated proteins and may function to drive the PB induction process (21,34). Results from the present study indicate that these proximal regions are not sufficient alone, or in conjunction with 0.8kb of surrounding 5'-flanking sequence, to mediate the PB induction response. Although it remains possible that this element may facilitate the PB activation process, in light of the current data it appears more likely that they constitute portions of the core promoter region of CYP2B2 and not specific PB enhancers or activators. Data to be presented in Chapter 2 indicate that the first 177 bases of CYP2B1 and CYP2B2 5' flanking sequence can promote PB-independent reporter gene transcription in a human hepatoma HepG2 cell line.

The results presented in the current study indicate a possible mechanism for PB induction in mammalian systems. The high degree of CYP2B2 constitutive expression in liver and kidney of the 19E mouse lines implies that levels of any requisite transcriptional activating factors in these tissues are not limiting. Since CYP2B2 transcription is largely repressed in untreated rat liver and other tissues, it may be hypothesized that the CYP2B2 gene is normally repressed by one or more negative regulatory factors and that PB functions to

uncouple such factors from their repressor role. Based on the 39E transgene results, negative control appears to be conferred by a repressor region between -0.8kb and -20 kb. This repressor element is PB-reversible and is perhaps selectively functional in the liver. Over this large span of sequence it is foreseeable that other elements also may be involved in gene control, *e.g.* a glucocorticoid responsive element has been identified (37), and DNase I footprint experiments demonstrate the presence of other protein binding motifs distal to the promoter (Chapter 3).

Although involvement of a barbiturate-modulated repressor protein has been similarly proposed for CYP102 regulation in *B. megaterium* (38), notable differences in PB induction mechanisms appear to exist among organisms. For example, comparatively high concentrations of barbiturate are required for CYP102 transcriptional activation in *B. megaterium*, approximately 1 order of magnitude greater than with vertebrate hepatocyte systems (36,39). A further disparity is that barbiturate induction of CYP102 in *B. megaterium* (38) and of CYP2B1 and CYP2B2 in the rat (40) is blocked by the protein synthesis inhibitor cycloheximide, whereas in the chicken, cycloheximide treatment alone leads to induction, and in combination with PB, superinduction of the barbiturate-inducible chicken P450, CYP2H1 (39). Thus, although PB responses may involve a common pathway, several apparently divergent mechanisms will need to be addressed in future studies.

DNA sequence data have not yet been reported for the 5' flanking regions of the CYP2H1 gene, thus it is unclear whether this gene possesses elements analogous to the 17- or 32-bp sequences defined by Fulco and Padmandaban, respectively (21,34). A 4.8-kb 5'-flanking region of the CYP2H1 gene was reported to possess PB enhancer-like activity in SV40 heterologous promoter constructs when transfected and expressed in primary cultures of chick embryo hepatocytes (39). In conjunction with the endogeous CYP2H1 promoter, however, this same region conferred only minor (2.4-fold) PB induction effects (39).

In planning these investigations, it was important not to rule out the possible location of important transcriptional regulatory sites for CYP2B2 existing at regions other than the proximal 5'-flanking domain. For example, the first intron of the human collagen a1 gene has been shown to contain a transcriptional activator (31), and the human β-globin gene possesses a DNaseI-hypersensitive site together with a tissue-specific activator in its

corresponding 3'-flanking domain (32). Furthermore, the mouse and human albumin genes exhibit transcriptional enhancer elements at -10 and -6kb, respectively, relative to their transcription initiation sites [Pinker, 1987 #103; Hayashi, 1992 #104]. However, the results of the current studies suggest that for the CYP2B2 gene, one or more regions upstream of -.8kb in the 5'-flanking region contain the primary elements contributing to PB induction and tissue-specific regulation. Further experiments are in progess to map these respective elements more precisely and to more thoroughly characterize the regulatory aspects of these transcriptional activation processes.

MATERIALS AND METHODS

TRANSGENIC MICE.

Constructs for transgenic mice were prepared previously by others in the lab and are diagrammed in Figure 1. Microinjections and oocyte implantation were performed by the University of Washington's Department of Comparative Medicine transgenic cost center program. DNA from mouse tails was checked for the presence of the transgene by Southern blotting and probing with the α-32P-labeled *Xba*I-*Acc*I fragment (41). Copy number was estimated by autoradiographic analysis carried out with a Molecular Dynamics model 425 PhosphorImager by the Imaging facility of the Markey Molecular Medicine Center at the University of Washington. DNA from Sprague-Dawley rats was used as a standard with two CYP2B2 gene copies. Founder mice were crossed to C57BL/6J mice, and F1 animals bearing the transgene were identified by PCR using primers E-621FP (5'-CCC ACC TGT GGC TAT CAT GG-3') AND E-28RP (5'-CTG AAT CTG CCC CTA CAC TC-3') using standard conditions (35). For phenobarbital treatments, mice 6-8 weeks of age were injected intraperitoneally with 80 mg/kg phenobarbital in saline 17 hours prior to liver extraction. Saline injections alone were administered to controls.

RNA AND PROTEIN ANALYSIS

RNA was isolated by the acid guanidium thiocyanate-phenol-chloroform method (42). For Northern blot analysis, 10μg of RNA was electrophoresed in 1.25% agarose gels containing 2M formaldehyde, transferred to membranes, and hybridized at 54°C to γ -^{32}P-

labeled oligomers as described (35,36). RNA slot blots were prepared using standard techniques. The discriminatory CYP2B1 oligomer, P450b, previously used and characterized for the rat (15,35,36) was exactly complementary to mouse CYP2b9 and used to probe endogenous mouse gene induction. The P450e oligomer (15,35,36), specific for rat CYP2B2 mRNA, was used to monitor expression of the rat transgene mRNAs in mouse tissues. Microsome purification and Western blot analysis were performed as described previously (15). A CYP2B1/2B2 antibody used to probe Western blots of the corresponding proteins was provided by Dr. P. Thomas of Rutgers University.

CHAPTER 2: IDENTIFICATION OF THE CYP2B1 AND 2B2 PROMOTERS AND UPSTREAM PROTEIN BINDING SITES.

SUMMARY

Non-PB inducible HepG2 cell lines were transfected with various chimeric CYP2B1 and CYP2B2 deletion mutant constructs. Analysis of CAT gene expression revealed the presence of a number of repressor elements upstream of their promoters; at least two are between -2497 and -802 in CYP2B2. Several protein binding elements within the promoters share identity with consensus transcription factor sequences. Identical DNA interactions occur in both control and PB induced states. The same is true for protein binding sites identified in DNA sequences surrounding a DNase I hypersensitive site at -1400bp.

INTRODUCTION

In chapter 1 it is reported that deletion of sequences 5' to -0.8kb removes the PB induction requirement for the CYP2B2 gene expression in transgenic mice, indicating that elements within the first 800bp may function as promoters and modulators of gene expression, but are not primarily involved in the PB induction response. The PB induction mechanism likely involves the relief of a repressor activity located 5' of -0.8kb. It may therefore be possible to study cis-acting CYP2B1 and CYP2B2 sequences in non-PB inducible hepatoma cell lines, which are relatively easy to transfect with foreign DNA, simply by finding which DNA region relieves repression of the promoter. Initial transfection experiments along this line are described in the first part of this chapter.

The remaining portions of this chapter are concerned with identifying protein interactions within two of three DNA regions of interest to our laboratory in the CYP2B1 and CYP2B2 genes. The first area of interest is the promoter region, by virtue of other reports, discussed in the previous sections, indicating its activity is modulated by PB. The second region is at around -1.4kb relative to the transcription start site. A DNase I hypersensitive

site maps to this position within the CYP2B1 and CYP2B2 genes, possibly indicating it is occupied by a DNA binding protein *in vivo* (see Appendix A). DNA in the vicinity of this hypersensitive site contains several consensus sequences of previously characterized transcription factor recognition motifs, as well as a functional GRE element in CYP2B2 (37). The third region of interest, at about -2.2kb, is a second DNase I hypersensitive site and was to be characterized by others in the lab.

RESULTS

TRANSIENT TRANSFECTION OF CYP2B1 AND CYP2B2 CAT CONSTRUCTS

A series of deletion mutants of CYP2B1 and CYP2B2 5' flanking sequence linked to a CAT reporter gene were constructed for previous experiments and were available for use in this assay (Figure 5, panel A). The ability of each construct to direct expression of the CAT gene in HepG2 cells was determined via transient CAT activity assays. In each of three separate studies, CYP2B1 and CYP2B2 constructs were introduced into HepG2 cells. A Rous Sarcoma virus (RSV) promoter-pBLCAT3 (RSVCAT) construct and a promoterless pBLCAT3 vector were used in each experiment as a positive and negative control, respectively. Mock transfections, having no plasmid DNA, were also performed and assayed to provide background CAT activity levels for each study. Plasmids used in this report were isolated simultaneously. An identical experiment using plasmids from a separate isolation gave analogous results. CAT activity levels, measured at 24 hours, were determined after subtraction of background levels. As expected, RSVCAT transfection resulted in high levels of transient CAT activity, while cells transfected with pBLCAT3 exhibited background levels. Successive 5' deletions beginning at -1394bp increased CAT activities of CYP2B1 constructs in a stepwise fashion (Figure 5, panel B). The deletion mutant -177CAT displayed maximum CAT synthesis among CYP2B1 constructs. Deletion of sequences -1394 through -550 moderately increased CAT activity 33% relative to RSVCAT, indicative of a negative element in this region. A stronger negative acting region was identified within -550 and -346 DNA, deletion of which resulted in a 2.6 fold increase in CAT activity. An additional 25% increase in CAT activity relative to RSVCAT was observed after a final deletion to -177bp, indicating the presence of a third negative region in sequence between -346 and -177 (Table II).

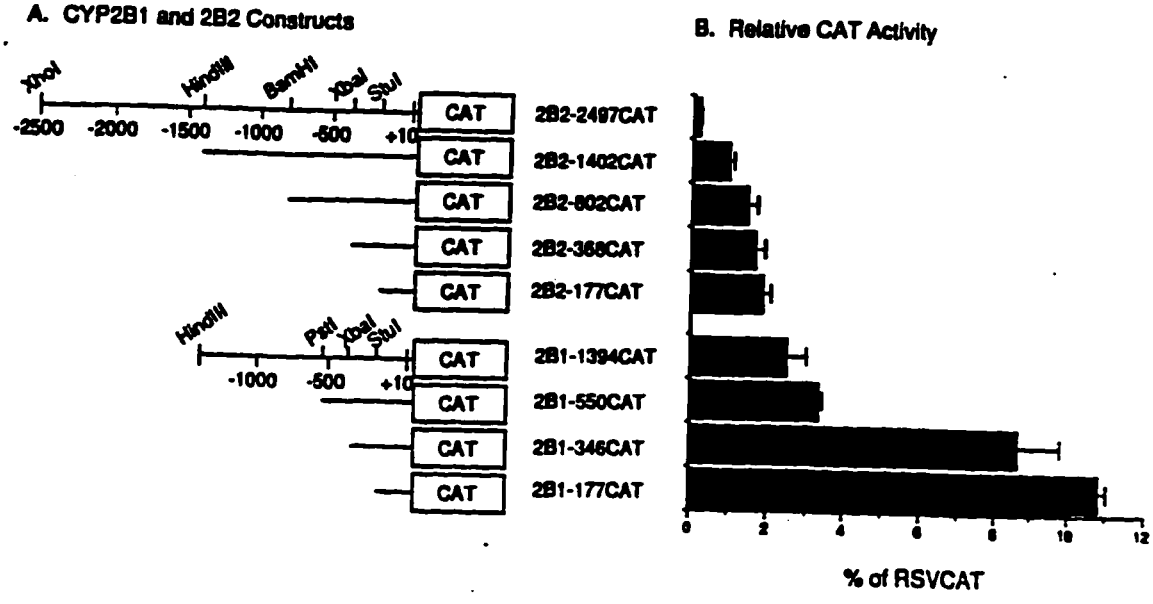


Figure 5. Transient CAT activity of CYP2B1 and CYP2B2 gene fusion constructs.

Panel A. Structure of CYP2B1 and CYP2B2 CAT plasmids used in transient CAT activity assays. Length of CYP2B1 and CYP2B2 5' sequence in each construct is presented in bp relative to the transcription start site. Restriction sites used in progressive 5' deletions are indicated along the longest construct for each gene. *Panel B.* Relative CAT activity of cells transfected with CYP2B1 and CYP2B2 deletion constructs. CAT activity levels of the respective plasmids diagrammed in Panel A are expressed as % of RSV-CAT activity. Values represent the mean of three independent assays. The standard deviation is indicated by error bars.

Table II. Values of CAT expression relative to RSVCAT

Mean and standard deviation *(s.d.)* values represent 3 separate experiments. The CAT activity of each construct relative to RSVCAT was calculated for each experiment to determine the *mean relative CAT activity*. The *% change in relative CAT activity* was calculated from the mean value determined for each construct. Each deletion resulted in an increase in CAT activity, indicated by a plus sign. The Student t-test was used to obtain the *p-value* for each successive deletion. *n.a.* =not applicable. *n.s.* = not significant

Construct	mean relative CAT activity	s.d.	% change in rel-- ative CAT activity	p-value
2B2-2497CAT	0.17	0.04	n.a.	n.a.
2B2-1402CAT	0.95	0.16	+458.8	<.01
2B2-802CAT	1.46	0.27	+53.7	<.05
2B2-368CAT	1.68	0.25	+15.1	n.s.
2B2-177CAT	1.87	0.21	+11.3	n.s.
2B1-1394CAT	2.51	0.53	n.a.	n.a.
2B1-550CAT	3.34	0.09	+33.0	<.10
2B1-346CAT	8.64	1.12	+158.7	<.01
2B1-177CAT	10.83	0.22	+25.3	<.05

Increasing CAT activity with decreasing 5' flanking DNA was also observed for CYP2B2 constructs (Figure 5, panel B). Like CYP2B1, CYP2B2 constructs with only the first -177bp of 5' sequence showed the greatest amount of CAT expression. However, CYP2B2-177CAT displayed over 5 fold lower CAT activity levels than the comparable CYP2B1 construct (Table II). Due to the lower levels of CAT synthesis directed by CYP2B2 containing vectors, modulations in CAT activity observed upon deletion of CYP2B2 5' sequence were not significant in some cases. Significant results were obtained with the first two deletion mutants, probably due to the larger magnitude of difference in CAT activity they effect. Deletion of the CYP2B2-2497CAT to CYP2B2-1402CAT resulted in a 5.5 fold increase in CAT activity. This increase in activity was the largest among all deletion constructs studied, and may be indicative of a strong negative cis-acting element residing between -2497 and -1402bp. Further deletion to -802bp resulted in a significant 54% increase in CAT activity.

ANALYSIS OF PROTEIN-DNA INTERACTIONS WITHIN CYP2B1 AND CYP2B2 5' FLANKING SEQUENCE

In vitro DNase I footprinting and electrophoretic mobility shift assays (EMSAs) were performed to determine protein-DNA interactions occuring within the promoter and at an upstream *in vivo* DNase I hypersensitive site at -1400. Of particular interest in these assays was to ascertain whether protein binding elements were differentially occupied by hepatic nuclear proteins in response to phenobarbital. To analyze protein binding sites of their promoters, a 187bp CYP2B1 or CYP2B2 fragment comprising -177 to +10bp from the transcription start site was 3' or 5' end labeled for use as upper or lower strand probes, respectively. Figure 6 shows the resulting footprint of the upper strand. Elements protected from DNase I digestion, indicative of protein binding, are numbered according to their position relative to the transcription start site. Despite the difference in activity levels of these two promoters, comparison of the banding patterns observed in DNase I footprints revealed no differences in protein-DNA interactions between CYP2B1 and CYP2B2. Similarly, samples incubated with control nuclear proteins gave identical results to those incubated with nuclear proteins from PB induced rats. Both promoters shared three protected regions. Table III shows a comparison of CYP2B1 and 2B2 protected elements

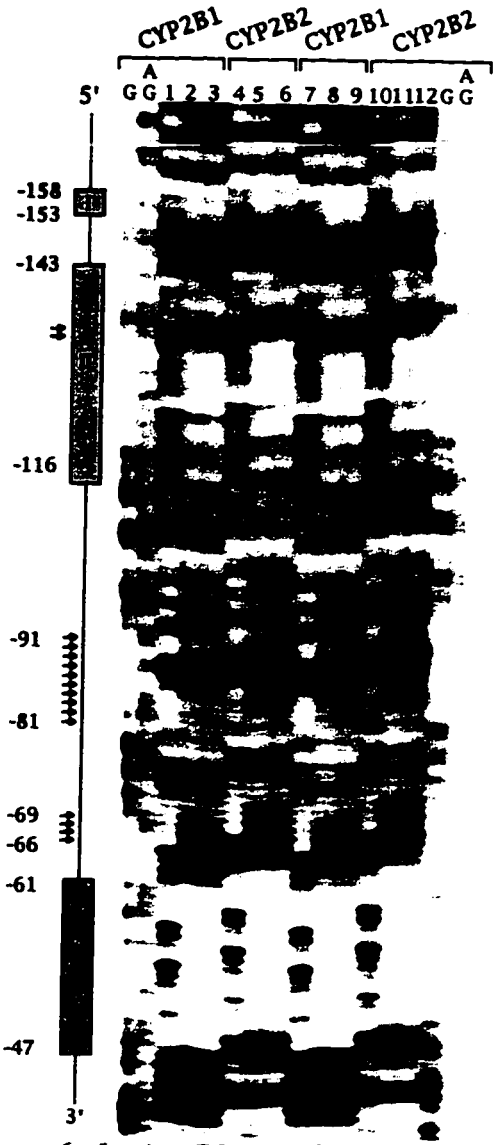


Figure 6. *In vitro* DNase I footprinting of the CYP2B1 and CYP2B2 gene promoter sense strand nucleotides

Probes were either *CYP2B1* or *CYP2B2* gene sequence from -177 to + 10 as indicated. Those incubated with DNase I in the absence of nuclear proteins correspond to lanes *1*, *4*, *7*, and *10*. Probes in lanes *2*, *5*, *8*, and *11* were incubated with control nuclear proteins, and those in lanes *3*, *6*, *9*, and *12* were incubated with nuclear proteins from PB induced rats. Samples contained either 1µg *(lanes 1-6)* or 10 µg *(lanes 7-12)* of non-specific competitor DNA poly dIdC. Protected regions *I*, *II*, and *III* are indicated by boxes, and DNase hypersensitive bases denoted by arrows. Positions of protected and hypersensitive bases were determined by Maxam and Gilbert *G* and *A+G* sequence

Table III. Comparison of protected sequences in the CYP2B1 and CYP2B2 genes with homologous consensus binding sites.

consensus HNF-5 element (43)	TGTTTGC/T	Footprint
CYP2B1/2B2 -144 to -138	TGTTTCG (5/7)	II
consensus C/EBP element (44)	GTGGTATGATTGC	II
CYP2B1/2B2 -138 to -126	GTGGTGTCCTTGC (10/13)	
consensus Oct-1 element (45)	ATTTGCAT	III
CYP2B1/2B2 -59 to -52	AGTTGCAT (7/8)	

with their corresponding consensus sequences. The first (protected element I) extends from -158 to -153 relative to the transcription start site. This protection was not uniform between different footprinting assays. Nucleotides just 5' of these bases are resistant to DNase I digestion, making it difficult to delineate the 5' boundary of protein binding. A protein binding site between -174 to -156 in the CYP2B2 gene was reported by others (46). The second protected region (protected element II) spans nucleotides between -143 and -116, with hypersensitive sites interposed at bases -135 and -134. The sequences protected here share identity with two recognition binding motifs to known transcriptional activators. At its 3' end is a C/EBP consensus element (44) at -138 to -126. Overlapping this element at the 5' end from -144 to -138 is an HNF-5 recognition motif (43). HNF-5 is a liver-specific factor found in enhancers or promoters of other hepatic genes such as rat tyrosine aminotransferase (43), hepatitis B virus (47), and human transferrin (48). A

number of overlapping HNF-5 and C/EBP motifs in other liver-specific genes such as rat tyrosine amino transferase (43) and hepatitis B virus have been identified (47). They share a single protected region from DNase I digestion with hepatic nuclear proteins in the human transferrin gene (48). Both C/EBP and HNF-5 elements generally act as positive modulators of transcription (45,49).

A third protected region (protected element III) in both CYP2B1 and 2B2 is located at -61 to -47. Within this sequence is a recognition element for the ubiquitous octamer binding protein Oct-1 located at -59 to -52 in the inverse orientation. Protected element III borders the overlapping BTE and 17b/e sequences previously identified as important cis acting elements in gel shift and in vitro transcription studies (19,21,22). Protein-DNA binding conditions were identical to those used to successfully bind both control and PB-induced nuclear protein extracts to the 17b/e oligomer in electrophoretic mobility shift experiments described below. Contrary to results obtained by others (34), nucleotides within the CYP2B1 and CYP2B2 BTE and 17b/e sites were not protected from DNase I digestion, despite multiple footprinting efforts using several distinct preparations of crude nuclear proteins. Instead, stretches of bases hypersensitive to DNase I digestion from -90 to -81 and from -69 to -66 surrounded these elements. *In vitro* footprinting performed on the lower strand of both promoters confirmed all of the above patterns of protected and DNase I hypersensitive sequences. A summary of these protected and hypersensitive bases by DNase I protection assays is presented in Figure 7, panel A.

Three different investigators comparing EMSAs of control and PB-induced nuclear proteins binding the 17b/e each reported dramatically different results. He and Fulco (21) showed 50-fold enhanced binding of PB extracts to the 17b/e oligonucleotide. Others, however, found equal binding between control and PB extracts to the CYP2B2 -104 to -71 fragment (19), or no binding by either extract to the 17b/e element (46). *In vitro* footprinting assays described above demonstrated protein-DNA interactions occur in both control and PB induced states. Accordingly, both control and PB-induced nuclear protein extracts showed significant protein binding to the CYP2B1 gene-specific 17b/e oligonucleotide in an EMSA (Figure 8). In this experiment it was observed that proteins from PB-induced nuclear extracts bound this element about 5-fold greater than those from control extracts.

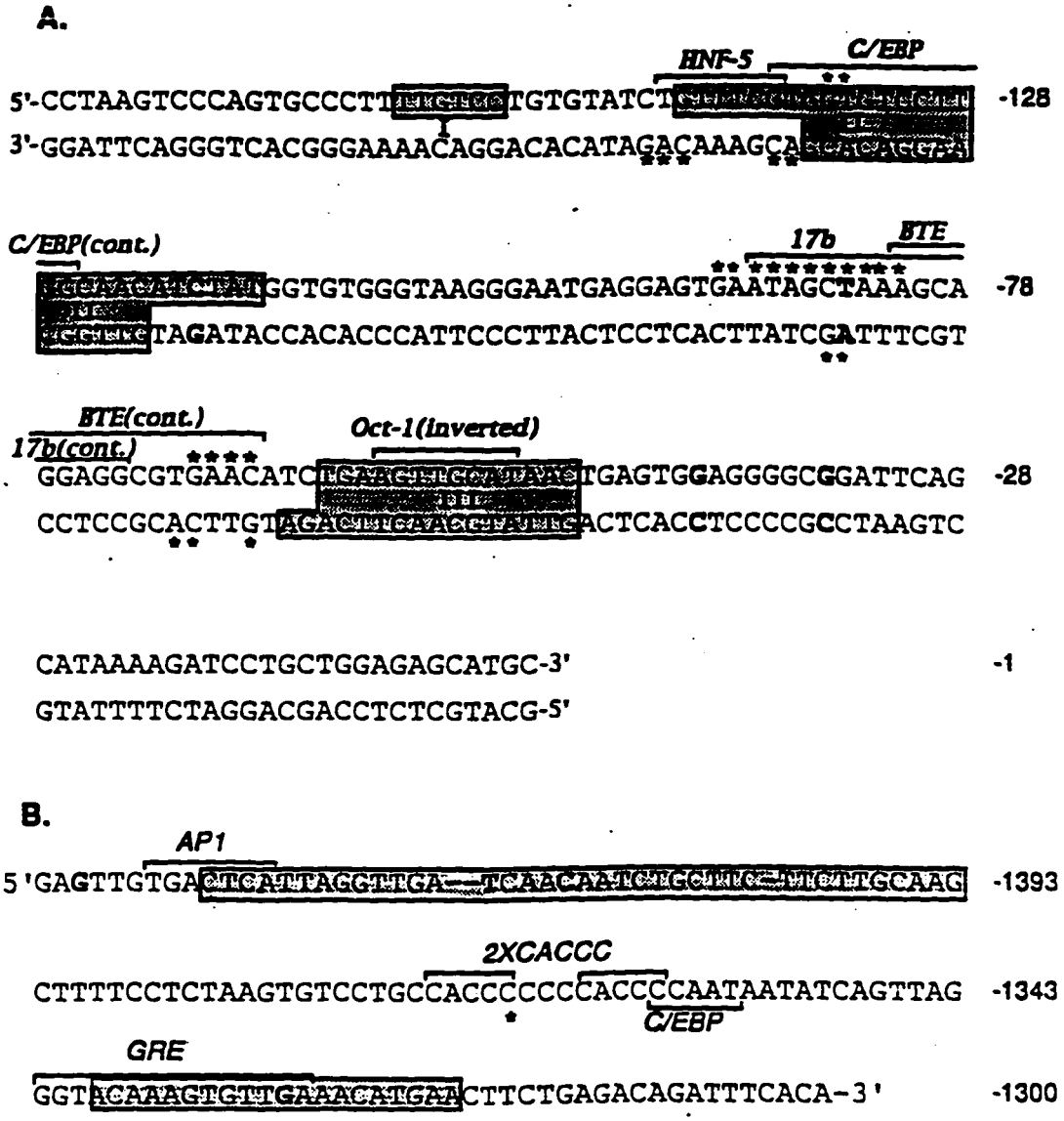


Figure 7. Summary of CYP2B1 DNaseI protected and hypersensitive bases

Panel A. The CYP2B1 promoter consisting of the first 177 bases of sequence 5' of the transcription start site. *Panel B.* CYP2B1 gene sequence from -1530 to -1300. DNase I hypersensitive bases are denoted by an asterisk. Consensus elements to known transcription factor binding sites are identified by brackets above their corresponding CYP2B1 sequence. Bases that are not homologous between CYP2B1 and CYP2B2 are printed in bold letters.

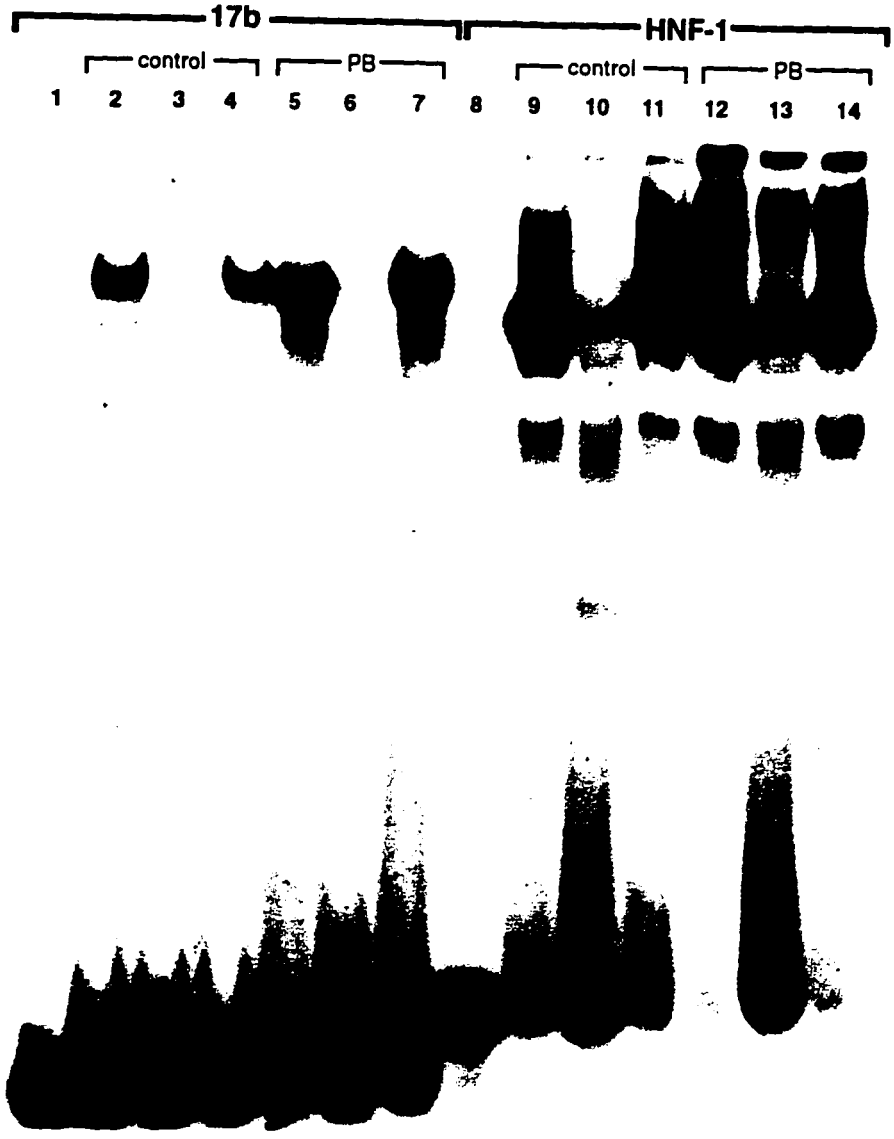


Figure 8. EMSA of 17b/e element with rat liver nuclear proteins

Double-stranded oligonucleotides in this assay corresponded to either the CYP2B1 specific *17b/e* or the rat albumin gene *HNF-1* element, run as a positive control. Probes were incubated without protein *(lanes 1, 8)* or with 3μg of nuclear protein from *control* or *PB* treated rats. Some samples were incubated with a 100-fold molar excess of the unlabeled probe *(lanes 3, 6, 10, and 13)* or 10μg of nonspecific competitor *poly dIdC (lanes 4, 7, 11, and 14).*

Protein binding sites were also characterized for a DNA region surrounding one of two sites hypersensitive to DNase I digestion *in vivo* (appendix A). *In vitro* footprints of control and PB-induced nuclear proteins bound to CYP2B1 gene sequence from -1500 to -1300 were determined (Figure 9). As with the promoter, footprints were identical between control or PB-induced nuclear proteins. Two protected regions were located at -1430 to -1388, and at -1338 to -1329. There is a DNase I hypersensitive site at -1367. The sequence protected at -1430 to -1388 is not similar to previously identified cis-acting regulatory elements. The region protected at -1338 to -1319 overlaps part of a glucocorticoid receptor recognition element (GRE) at -1343 to -1328. This GRE consensus sequence is almost identical (14 out of 15 bases) to that in CYP2B2 reported to enhance basal transcription in response to the GR ligand dexamethasone (37). The DNase I hypersensitive base at -1367 is part of a consensus CACCC element (50) and is immediately 5' of a C/EBP consensus binding motif (Figure 7, panel B).

DISCUSSION

In this chapter, the ability of proximal CYP2B1 and CYP2B2 flanking sequence to modulate CAT reporter gene expression in HepG2 cells has been analyzed. Because elements involved in the constitutive repression of these genes were to be examined, it was thought that PB inducibility may not be a requirement for a test cell line. Transgenic mice data presented in chapter 1 suggested a cis acting transcriptional repressor important in PB induction was 5' of -.8kb in the CYP2B2 gene. Since the 97% nucleotide sequence identity existing between the CYP2B1 and CYP2B2 breaks off at approximately -2.5kb (19), it is likely that important sequences regulating the PB induced expression of these genes are within DNA between -2.5 and -.8kb. Transfection data described in this chapter indicated the presence of at least two negative cis-acting regions meeting this criteria in the CYP2B2 gene sequence. By far the strongest repressive effect was directed by CYP2B2 DNA from -2497 to -1402. CYP2B2 gene sequence from -1402 to -802 only weakly repressed reporter gene transcription.

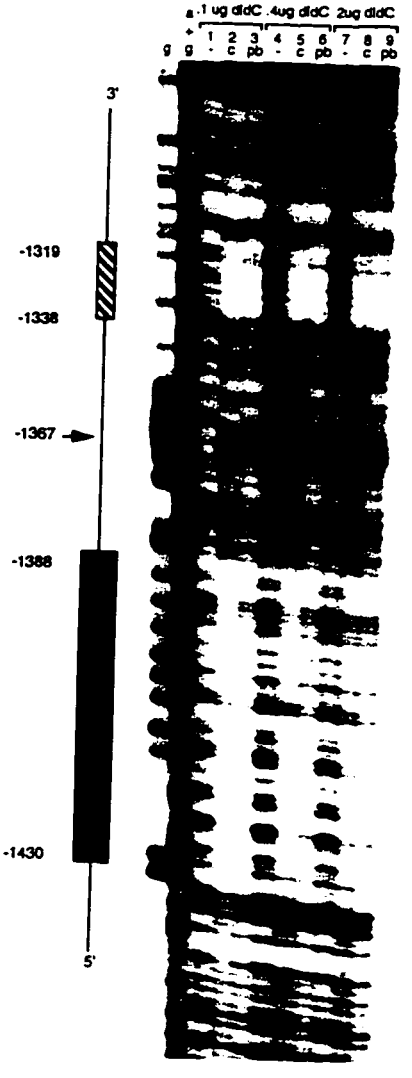


Figure 9. In vitro DNaseI footprint of CYP2B1 sequence between -1530 and -1200

The probe is a 330bp fragment of CYP2B1 gene sequence between -1530 and -1200, labeled at -1530 (lower strand) and incubated with either 0 protein *(lanes 1, 4, and 7)*, 20μg protein from control rats *(lanes 2, 5, and 8)*, and 20μg protein from PB-treated rats *(lanes 3, 6, and 9)*. Concentrations of *poly dIdC* used are indicated above each lane. The first two lanes are the corresponding Maxam and Gilbert *G* and *A+G* sequences of the labeled fragment. Vertical boxes correlate to regions protected from DNase I digestion; the arrow indicates DNase I hypersensitive bases.

These transfection experiments revealed that negative cis-acting elements are located within the first 802bp as well. Using sequential deletions of 5' flanking DNA, three negative cis-acting regions in CYP2B1 were identified. Two that only weakly repress transcription are within CYP2B1 5' sequence -1394 to -550 and -346 to -177. A third negative element was identified within the sequence spanning -550 and -346. Maximum CAT synthesis among all constructs surveyed was observed within the first 177bp of 5' flanking sequence. The analogous 177bp of CYP2B2 5' sequence was identified as a promoter via in vitro transcription assays by other investigators (19,22). CYP2B2 deletion mutants consistently expressed CAT at lower levels than comparable CYP2B1 constructs. This may have been due to the five-fold lower activity of its proximal promoter within the first 177bp of 5' flanking DNA (Figure 5). It is interesting to note that the endogenous CYP2B1 gene is expressed at 4 to 5 fold higher levels than CYP2B2 in PB induced Sprague Dawley rats (20,36). Similar transient transfection studies performed in Cos I cells showed identical trends in CAT activation as those in HepG2 (unpublished results). It is hypothesized that the multiple loci of repressor elements discovered in this assay may be a genetic strategy for ensuring the tight restriction of CYP2B1 and CYP2B2 expression under constitutive conditions. These experiments also suggest that non-PB inducible hepatoma cell lines may contain all of the elements necessary for basal promotion of these genes but are deficient in a mechanism to relieve upstream repression in response to PB.

The results obtained from *in vitro* DNase I footprinting assays correlate well with evidence implying that upstream DNA represses basal promotor activity in the CYP2B1 and CYP2B2 genes. Footprints of DNA binding proteins within the promoter, consisting of the first 177bp 5' of the transcription start site, were identical between probes incubated in hepatic nuclear extracts from control or PB-exposed rats. Protein-DNA complexes were also identical between the two promoters (CYP2B1 and CYP2B2) although there was a five fold difference in their ability to promote CAT activity. However, variations in the amount of protein binding to each element would not be detected here. At least three protein binding elements were observed in this assay. Elements consistently protected from digestion by nuclear proteins were similar in sequence to recognition motifs of constitutively expressed proteins that activate transcription in other liver-specific genes *e.g.* (48,51,52).

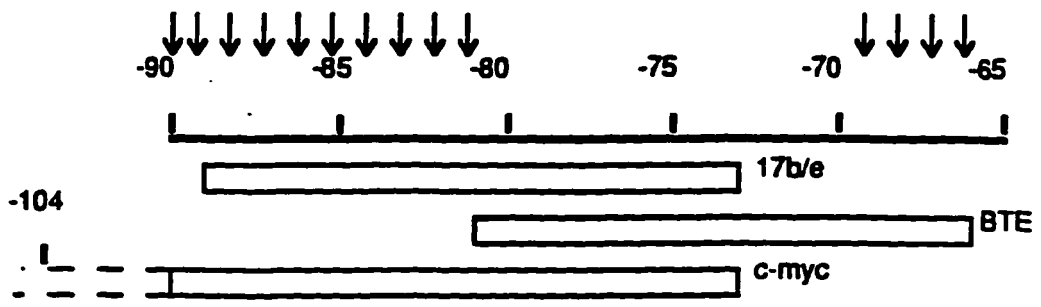


Figure 10. Summary of consensus sites reported for CYP2B1 and CYP2B2 gene sequence between -90 and -65

Arrows represent DNase I hypersensitive nucleotides identified in footprinting assays. The solid line represents CYP2B1 and CYP2B2 sequence between *-90* and *-65* bp 5' of the transcription start site. Boxes correspond to the positions of transcription factor binding sites that have been reported by others (19,21,34).

In addition to these protected regions, nuclear hepatic proteins increased the DNase I hypersensitivity of two nucleotide tracts. These hypersensitive regions border an area of particular interest as there is conflicting reports regarding its ability to bind protein and activate transcription in response to PB (19,21,22,46). BTE, an element involved in the basic transcription of dioxin-inducible CYP1A1 (53), is located between these two clusters at -81 to -66. Spanning the more distal hypersensitive stretch is the 17bp putative PB-responsive 17b/e or "barbie-box" element at -89 to -73 (21) (see Figure 10). According to EMSAs presented here, the 17b/e element does indeed bind more proteins from PB-induced animals than from uninduced. However, it is unlikely this directly effects an increase in transcriptional activation *in vivo* according to data presented in chapter 1. It is possible this element activates transcription indirectly via interactions with proteins binding upstream. Alternatively, increased binding here may actually down-regulate transcription activation in response to PB. Mice carrying the transgene with only the first -.8kb of 5' flanking sequence expressed lower levels of the CYP2B2 gene after PB treatment (chapter 1).

One of two positions in the CYP2B1 and CYP2B2 genes that is sensitive to DNase I cleavage *in vivo*, at about -1.4kb, is within DNA that constitutively repressed transcription of the CAT gene in transient transfection assays and is of course 5' of -0.8kb. Despite having these characteristics required of a PB-response element according to transgenic mouse experiments (chapter 1), protein binding elements in this region are unlikely candidates for this function. Proteins from control and PB-induced nuclear extracts displayed equivalent DNA-interactions with CYP2B1 gene sequence between -1500 and -1300 in *in vitro* footprinting assays. Data from subsequent transgenic mouse experiments, presented in appendix B, imply that the PB response is mediated by DNA sequence 5' of -1679. The position of putative transcription factor binding sites relative to *in vivo* DNase I hypersensitive sites are diagrammed in Figure 11.

MATERIALS AND METHODS

CELL CULTURE AND TRANSFECTION

HepG2 cells were maintained in DMEM:F12 (1:1), penicillin-streptomycin (P/S) (100U/ml) and 5% Nu-Serum (Collaborative Research). Cells were grown to confluency

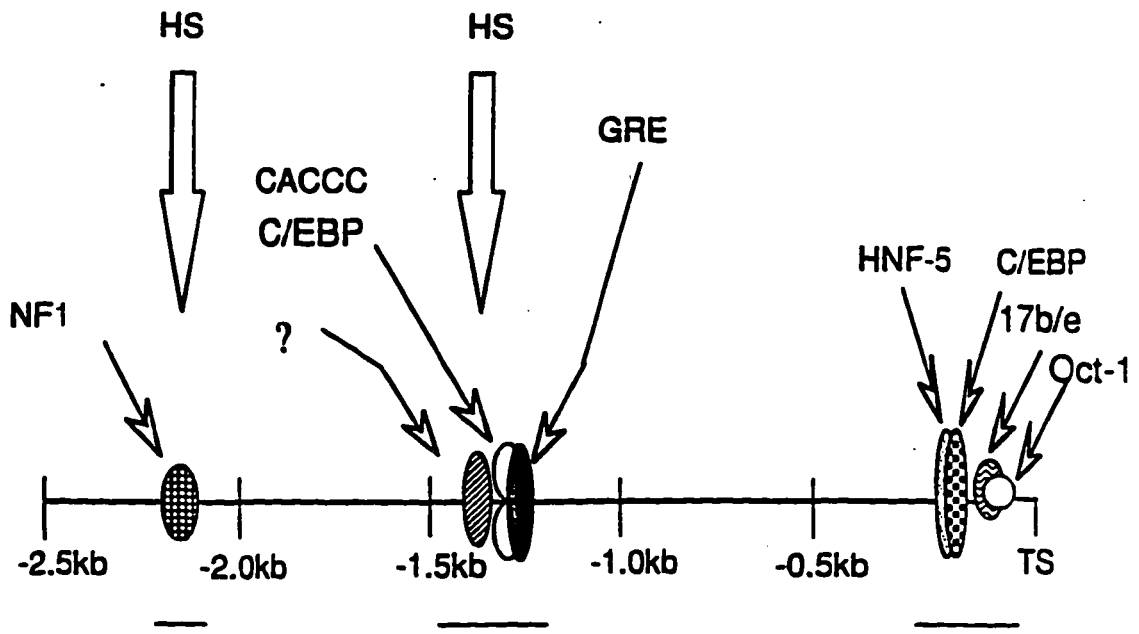


Figure 11. Putative transcription factor binding sites in CYP2B1 and CYP2B2 5' flanking sequence

Hepatic nuclear proteins interacting with consensus recognition motifs for known transcription factors *in vitro* are diagrammed as ellipses along the 5' sequence. All of these consensus motifs have been described in this chapter with the exception of *NF-1*, which will be described in chapter 3. The large arrows show the position of two *in vivo* DNase I hypersensitive sites *(HS)*. The lines along the bottom of this figure correspond to DNA regions that have been assayed by *in vitro* footprinting. *TS* = transcription start site.

and passed 24 to 48 hours prior to transfection. At 30 to 40% confluency, transfections were carried out using a lipofectin method (54). Plasmid DNA, isolated as described in (55) (2.5pmol/ml) in each sample was transfected for 6 hours after which the medium was changed. Cells were cultured for 48 hours with a single medium change at 24 hours post transfection.

CAT ASSAYS

CAT activity assays were performed as described (56). The Student t-test was used to test significance of changes in CAT activity between successive deletions.

PREPARATION OF CRUDE NUCLEAR PROTEINS

Three month old Sprague-Dawley rats were injected with PB (80mg/kg). Twenty hours post injection, liver nuclear proteins from PB-induced and control rats were isolated (57). The induction status of PB treated rats was confirmed by Northern blot analysis, as previously described (41).

DNASE I FOOTPRINTING

To footprint the CYP2B1 and CYP2B2 gene promoters, plasmids (CYP2B1-350,+10) pKS and (CYP2B2-369,+10) pKS were digested with *Xho* I, followed by a Klenow fill-in (sense strand) or polynucleotide T4 kinase (antisense strand) labeling reaction. The single end labeled fragments were removed from the plasmid by digestion with *Sac* II, followed by gel purification. To obtain footprints of a putative upstream protein binding element plasmid (CYP2B1-1480, -1180) pKS was cut with *Xba*I, radiolabeled with a Klenow fill-in reaction, then cut with *Eco*RV and gel purified. DNase I footprinting was performed as described (58) with modifications. In a 100µl volume, a buffer consisting of 10mM HEPES-OH (pH 7.6), 50mM KCl, 0.1mM EDTA, 10% glycerol, 4mM $MgCl_2$, 1mM $CaCl_2$, 50µg/ul BSA, 5mM DTT and 1 or 10µg poly dIdC was incubated 15 minutes on ice with or without 20µg of liver nuclear proteins from control or PB-treated Sprague-Dawley rats. Radiolabelled probe (15,000 cpm) was added and incubated an additional 15 minutes at room temperature, followed by a one minute DNase I treatment using 0.01µg (samples with no protein) or 0.2-0.75µg (samples with protein) DNase I. The reaction was stopped

by adding 100µl of DNase I stop buffer (50mM EDTA, 0.2% SDS, 100µg/ml tRNA and 100µg/ml proteinase k) and incubated at 42°C for 45 minutes, then extracted with PCI and precipitated overnight with ethanol. Each reaction was suspended in loading dye and separated on a 6% denaturing polyacrylamide gel followed by autoradiography.

ELECTROPHORETIC MOBILITY SHIFT ASSAY

EMSAs were performed as described by Ausubel et al. (58) using the following buffer conditions: 60mM KCl, 1mM EDTA, 12mM HEPES (pH 7.9), 1mM DTT, 4mM Tris-HCl (pH 7.9), 300µg/ml BSA, 12% glycerol, and 1µg of non-specific competitor DNA poly dIdC. Samples were electophoresed on a low-ionic strangth 6% polyacrylamide gel as described (58).

CHAPTER 3: A NF-1 FAMILY MEMBER BINDS A FAR UPSTREAM REGULATORY ELEMENT IN CYP2B2 GENE

SUMMARY

A CYP2B2 gene sequence at -2.2kb that binds rat hepatic nuclear proteins both *in vitro* and *in vivo* was identified previously by others in the Omiecinski laboratory. Although it is within the sequence required for transcriptional activation in response to PB, hepatic nuclear proteins occupy this site regardless of induction status. An increase in protein binding is observed after PB exposure. This DNA binding protein was identified as NF-1 on the basis of its recognition sequence specificity and immuno-reactivity. The major NF-1 form retained after DNA binding-site affinity purification was 32kDa, the approximate size of the NF1-L protein that has been cloned by others (59). The observed increase in binding of this NF1-L protein to CYP2B2 -2271 to -2186 after PB induction was not correlated with NF1-L transcript levels or gross phosphorylation status as determined by immuno-reactivity with antibodies raised against phosphorylated amino acids.

INTRODUCTION

A protein binding element was identified between -2221 and -2197 in the CYP2B2 gene by Richard Ramsden in the Omiecinski laboratory, as described in Appendix A. Sequence within this region is nearly identical to the nuclear factor 1 (NF-1) recognition motif (87%), and moderately identical to that of NF-κB (67%). In all of the assays used to identify this protein binding site, described in appendix A, protein-DNA interactions occured both with and without PB induction. However, in protein filter binding assays, DNA fragments containing the above element were consistently retained on the filter in larger amounts by PB induced nuclear extracts compared to control. This protein binding site is within a 170bp CYP2B2 gene fragment that directed moderate PB-inducible reporter gene expression in transient primary hepatocyte transfection assays reported by other investigators (60). It is also contained within the -2497/-1679 CYP2B2 fragment that

appears to confer PB inducible reporter gene expression in transgenic mouse experiments (appendix B). These data have led us to believe this may be an important cis-acting sequence in PB induction. This chapter describes efforts undertaken to characterize the protein binding at this recognition element.

RESULTS

CHARACTERIZATION OF UPSTREAM DNA BINDING PROTEIN RECOGNITION SEQUENCE

Electrophoretic mobility shift assays (EMSAs) were performed as an initial characterization of the upstream element binding protein. Incubation of the radiolabeled probe -2271/-2186 CYP2B2 with hepatic nuclear extracts from control or PB-treated rats produced equivalent patterns of 6 shifted bands relative to the free probe. This suggested the presence of either 6 distinct DNA-protein interactions, a DNA binding protein with multiple protein-protein interactions, or a combination of these two possibilities. In order to discriminate among these possibilities, EMSAs were run with an increasing amount of KCl in the binding reaction. In these assays protein-DNA binding interactions were most intense in the presence of 150mM to 200mM KCl. Higher or lower KCl concentrations resulted in weaker DNA binding, albeit uniformly for all 6 bands, a result suggestive of discrete DNA-binding proteins (61). A substantial amount of DNA binding was evident in the presence of up to 1M KCl (Figure 12).

EMSAs of the radiolabeled -2271/-2186 CYP2B2 probes were also performed in the presence of competing unlabeled synthetic double-stranded oligonucleotides. Competitor oligonucleotides were designed with the intention of distinguishing if the DNA binding proteins were specific for NF-1 or NF-κB recognition sequences. As expected, a 34bp oligonucleotide corresponding to the CYP2B2 element region which protected in *in vitro* footprinting assays uniformly competed away all protein-DNA interactions. Alternatively, an identical 34bp oligonucleotide, with point mutations introduced at two G residues in

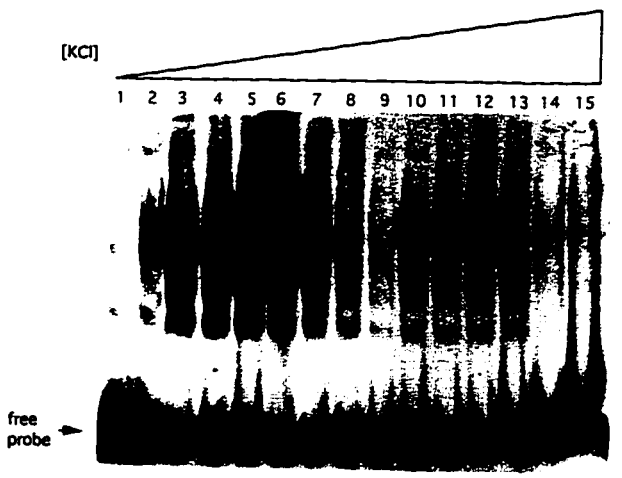


Figure 12. Effect of increasing KCl concentrations on protein binding to -2271 to -2186 CYP2B2 gene sequence

For each sample, 1ug of control nuclear protein extracts was incubated with the -2271 to -2186 probe under identical binding conditions with the exception of KCl concentration. KCl concentrations between lanes *1* and *15* are as follows (in order): 50mM, 75mM, 100mM, 150mM, 200mM, 300mM, 400mM, 500mM, 600mM, 700mM, 800mM, 900mM, 1M, 1.5M, and 2.0M.

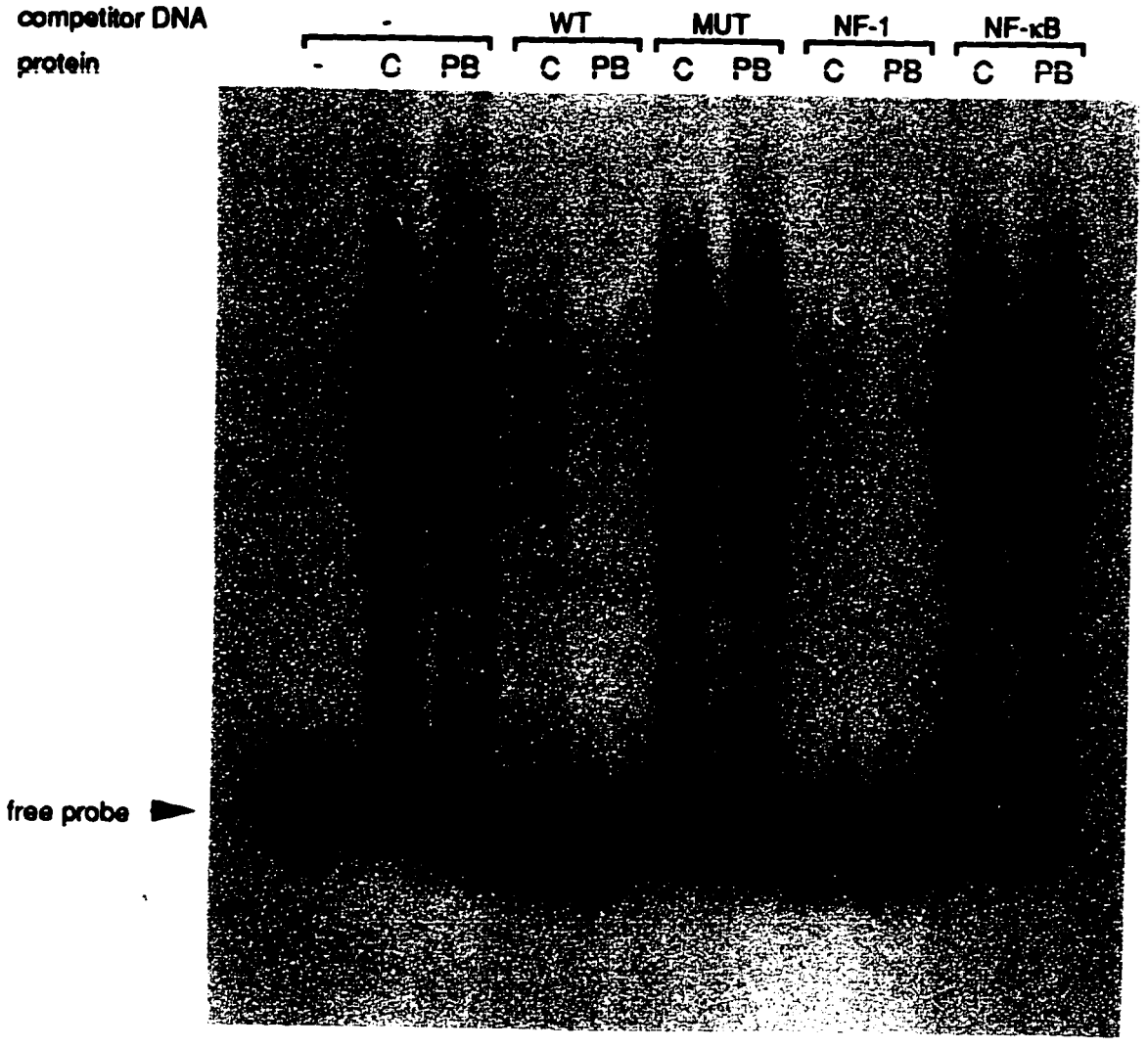


Figure 13. Electrophoretic mobility shift assay of a -2271 to -2186 CYP2B2 gene probe

Interactions between 1ug of control (*C*) and PB-induced (*PB*) nuclear protein extracts with the -2271 to -2186 gene probe were examined in the presence and absence of competing recognition sequences. The migration of the free probe, incubated without added protein (-) is indicated by the lower arrow. (-) = no competitor added; *WT* = wild-type CYP2B2 protein-binding element; *MUT* = CYP2B2 protein-binding element mutated at 2 G residues; *NF-1* = NF-1 consensus oligonucleotide; *NF-κB* = NF-κB consensus oligonucleotide.

positions critical for NF-1 binding (62,63; see Materials and Methods) was unable to compete for any of the observed DNA binding proteins. Likewise, all 6 protein-DNA binding interactions were competed away by a 21bp NF-1 consensus binding element, while they were all unaffected by a 22bp NF-κB consensus sequence. Identical results were achieved with hepatic nuclear proteins from control or PB exposed rats.

AFFINITY PURIFICATION OF PROTEINS BINDING UPSTREAM DNA ELEMENT.

Initial attempts to immunologically identify the DNA binding proteins binding in the EMSAs described above were unsuccessful. Because western blot analysis suggested that both NF-1 and NF-κB existed in very low abundance compared to other transcription factors (such as C/EBP) in our nuclear protein fractions (data not shown), magnetic DNA affinity beads were used as a means to enrich protein samples for the specific -2271/-2186 CYP2B2-binding protein. In this procedure, DNA fragments containing the high affinity protein binding site attached to magnetic beads were incubated with equal amounts of either control or PB-induced crude nuclear protein extracts at optimal salt concentrations for protein-DNA interactions, as identified in EMSAs. Specific DNA binding proteins could then be physically removed from the remaining supernatant by an externally applied magnet, rinsed with non-specific competitor DNA poly dIdC, and eluted in high salt.

This procedure was performed for three distinct nuclear protein extracts from both control and PB-induced states. The first two sets of extracts (each set was composed of both control and PB-induced nuclear protein samples) were derived from rat livers excised sixteen hours after control or PB-treatment, about when CYP2B2 transcripts are most abundant (10), while the third set was obtained four hours post-injection, when transcription initiation events are thought to peak (9,10). In all cases, total protein stains of SDS-PAGE gels revealed that the major eluted protein species migrated at approximately 32kDa as can be seen in Figure 14. Although present in both control and PB-induced extracts, proteins of this size were generally more abundant in the latter. Interstingly, a 30kDa protein band running just beneath the 32kDa protein is present only in PB-induced proteins obtained at the 4 hour time point, but is found in both control and PB-induced extracts at 16 hours. A second protein found in relatively high abundance, and in quantities independent of induction status, had a molecular weight of approximately 88kDA. The

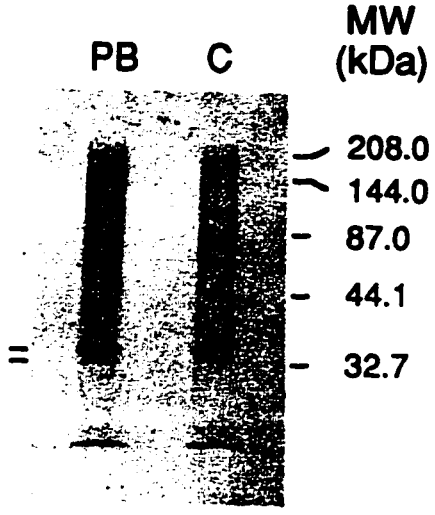


Figure 14. Total protein stain of binding site affinity purified nuclear proteins.

Rat liver nuclear proteins obtained 4 hours after saline control (*C*) or PB (*PB*) injections were purified on the basis of their affinity for the -2271 to -2186 CYP2B2 protein binding element as described in "Materials and Methods". The eluate (27µl) was analyzed via SDS-PAGE followed by coomassie blue staining. Bars to the left of the figure are positioned next to the two proteins that were present in greater amounts with PB treatment.

presence of multiple minor protein bands represents contamination by non-specific DNA binding proteins, a result not unexpected for affinity purification directly from crude extracts (64).

Immunodetection with antibodies directed against NF-1 revealed the presence of eight immunologically reactive proteins, all present in equal amounts between control and PB-induced samples A representative immunoblot of one set of samples is shown in Figure 15, panel A. Proteins recognized by the NF-1 antibody have approximate molecular weights of 140 , 130, 120, 88, 70, 60, 50, and 32kDa. NF-1 proteins at 32 and 88kDa match the observed size of the two dominant eluted proteins discussed above; quantities of the 32kDa protein appeared equal in control and PB-induced proteins. Densitometry analyses, comparing the 88kDa NF-1 protein ECL signal intensities before and after affinity purification demonstrated a roughly 50-fold enhancement of this NF-1 protein per µl protein sample. The 32kDa NF-1 protein was below the limit of detectability in our crude extracts. Conversely, NF-κB levels decreased by about an estimated 25 fold per µl, and were not modulated by PB status. Immunoblots with anti-phosphotyrosine, and anti-phosphothreonine antibodies did not reveal any alterations in protein phosphorylation status with PB induction. However, a low molecular weight protein (<17kDa), that did not cross react with the NF-1 antibody, was detected with the anti-phospho-serine antibody selectively in control affinity purified samples (Figure 16).

Affinity purification was also performed on a fourth set of nuclear protein extracts harvested from primary rat hepatocytes four hours after exposure to control or PB-containing media. To remove non-specific or low-affinity DNA binding proteins here, highly stringent washing conditions were used, including: Increasing the number of ten-fold molar excess of non-specific competitor DNA washes from 6 to 10; using a more efficient competitor, calf thymus DNA, instead of poly dIdC; and including an extra wash with 400mM KCl prior to elution. Immunoblots of the resulting eluate revealed the absence of proteins recognized by anti-phosphotyrosine and NF-κB antibodies. Additionally, only one of the NF-1 immunoreactive proteins were retained under these rigorous conditions. As Figure 15 panel B shows, it had a molecular weight of 32kDa and appeared only in the two distinct extract samples derived from PB-induced hepatocytes.

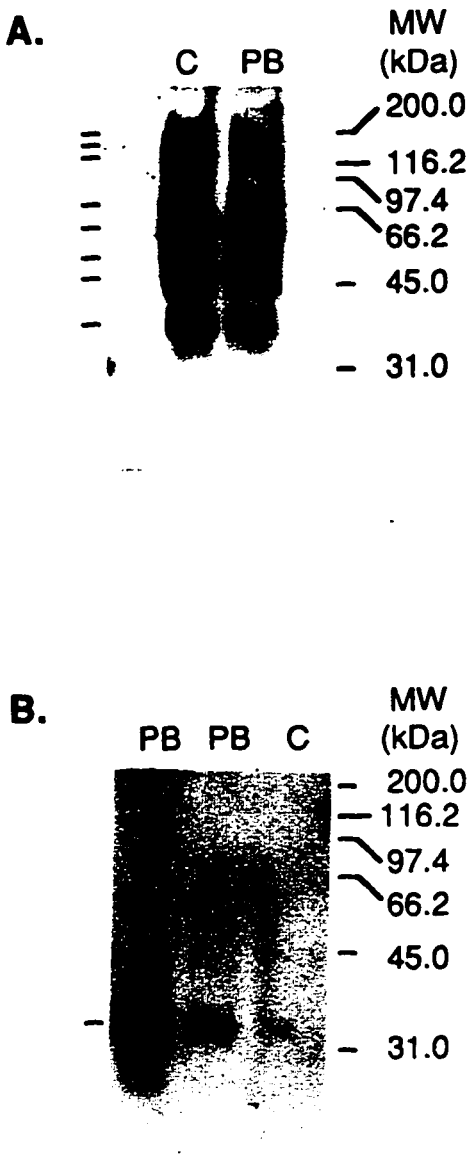


Figure 15. Detection of NF-1 family members in affinity purified nuclear proteins.

Affinity purified proteins derived from: *Panel A*. rat liver nuclei 16 hours after saline control (*C*) or PB (*PB*) injection; *Panel B*. primary rat hepatocyte nuclei 4 hours after exposure to control or PB-containing medium. Western blot analyses using the NF-1 antibody were performed on portions of the resulting samples (27µl) as described in "Materials and Methods". Bars to the left of each autoradiogram indicate positions of immunoreactive protein bands.

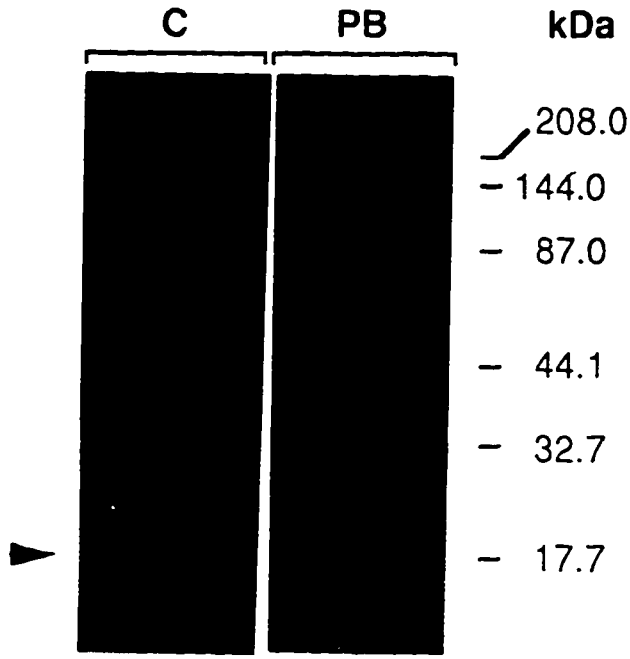


Figure 16. Immunoreactivity of affinity purified proteins to anti-phospho-serine antibody.

Western blot analysis of two samples of affinity purified protein from control *(C)* or PB-treated hepatic nuclear extracts *(PB)* probed with an antibody against phospho-serine residues. The line to the left of the blot shows the position of the protein selectively phosphorylated in control samples. The migration of molecular weight standards *(MW)* is shown at the right.

COMPARISON OF NF1-L TRANSCRIPT LEVELS BEFORE AND AFTER PB EXPOSURE.

The cDNA sequence encoding a NF-1 family member found in rat liver (NF1-L) has been previously reported, and a rough estimate of its molecular weight is about 30kDa (59). The amount of this and other NF-1 proteins, modulated at the level of transcription, are sensitive to changes in the cellular environment (65-67). To account for increases in the amount of the 32kDa protein obtained from PB-induced affinity-purified extracts described above, the quantity of NF1-L transcripts in the polyA+ fraction between control and PB-induced rat livers were compared. NF1-l mRNA levels were equivalent between control and PB-induced states for four sets of animals (Figure 17).

DISCUSSION

The experiments described in appendix A demonstrated the presence of a DNA binding protein in hepatic nuclei whose recognition sequence resides 2200bp 5' of the transcription start site in the CYP2B1 and 2B2 genes. A DNA-binding protein appears to occupy this site regardless of gene activation status in all of the *in vitro* assays presented in this chapter and in appendix A, as well as *in vivo* in DNase I hypersensitive site mapping experiments. Competitive EMSAs and western blot analyses of DNA affinity purified proteins both demonstrated that at least six distinct forms of the transcription factor NF-1 can bind this site with a high affinity.

NF-1 proteins belong to a family of DNA-binding proteins with diverse functions in transcription promotion, enhancement, repression, and in replication of the adenovirus DNA. Within a species, an array of NF-1 proteins are expressed on a tissue-specific basis (59,68). These can be derived from distinct NF-1 genes and from splice variants of these gene products (69,70). Adding further complexity to the study of these proteins, NF-1 may bind its recognition element as either a homodimer (71) or a heterodimer with other NF-1 forms (63). All NF-1 family members, within and between species, share a highly

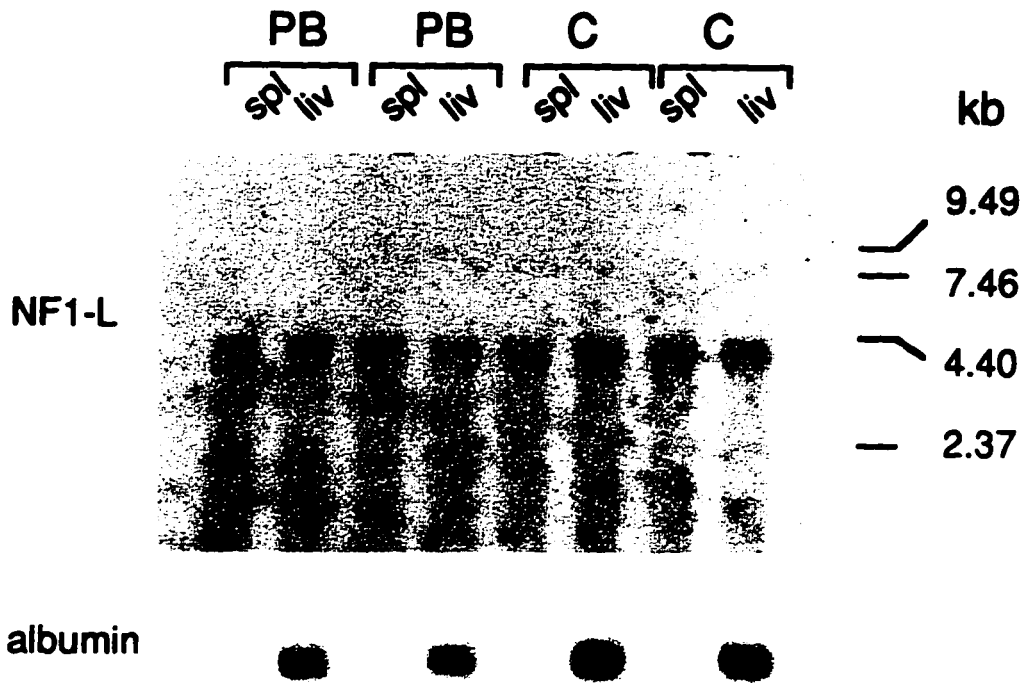


Figure 17. Northern blot assay of NF1-L in poly A+ RNA obtained from liver and spleen of control PB-induced Marshall rats

Poly A+ RNA was extracted from the spleen *(spl)* and liver *(liv)* of two control *(C)* and two PB-treated rats *(PB)*. Blots were probed with the NF1-L specific cDNA probe *(NF1-L)* , then stripped and re-probed with an albumin oligonucleotide probe *(albumin)* as described in Materials and Methods. The position of RNA size standards (in *kb*) is indicated to the right of the autoradiogram. Density of NF1-L autoradiogram signals were normalized to those from albumin for each sample.

conserved amino terminal domain required for DNA binding, protein dimerization, and DNA replication (71). The carboxy-terminal domains involved in transcription activation, however, are quite divergent. Several studies report that individual NF-1 forms may have unique transcription activation properties (72-74). It has also been reported that the nucleotides surrounding the NF-1 consensus sequence and within the variable region can influence NF-1 binding affinity (75) and may perhaps direct preferential binding to a unique NF-1 form (74).

In EMSA assays reported here, the CYP2B2 NF-1 element binds six distinct proteins in crude nuclear protein extracts. Analyses of DNA affinity purified extracts suggested a 32kDa NF-1 form may be of particular importance here. This NF-1 protein is selectively retained on the CYP2B2 cognate binding element under stringent washing conditions, and is the identical size of the most abundant protein derived from affinity purification. This protein also displayed a higher binding activity for the CYP2B2 NF-1 element after PB exposure, a result seen in protein filter binding assays of crude extracts (appendix A), and reported by others (60). It is unlikely this PB-induced increase in DNA binding activity in itself would initiate the CYP2B2 PB response, as the same protein binds DNA when the gene is transcriptionally inactive. Northern blots hybridized with an NF1-L specific probe indicate that the observed increase in DNA binding activity is not a result of PB induction of the NF-1 transcripts putatively encoding the protein of interest. It is possible that the observed alteration in DNA binding affinity reflects a conformational change in the NF-1 protein induced by PB exposure. An increase in DNA binding affinity may also be secondary to effects elsewhere in the NF-1 protein, such as interactions with co-activator proteins or conformational changes in the activation domain.

Previous experiments have suggested the involvement of a phosphorylation pathway in mediating the PB response. Specifically, addition of cAMP agonists to PB-containing media completely obliterated induction of the CYP2B1 and 2B2 genes in cultured rat primary hepatocytes (76). The cell contains a number of nuclear protein kinases and phosphotases acting as downstream effectors of signal transduction phosphorylation cascades. These act to alter gene expression in response to extracellular stimuli through covalent modification of certain transcription factors (77). PB exposure had no effect on tyrosine, threonine, or serine phosphorylation of affinity purified NF-1 proteins that could be immunologically detected. This method of detecting amino acid phosphorylation,

however, is not likely to be sensitive enough to detect changes in phosphorylation of a single residue. A low molecular weight protein, immunologically distinct from NF-1 but co-eluting with NF-1 during binding site affinity purification, was selectively detected with an anti-phosphoserine antibody in control hepatic nuclear extracts. Nancy Beck in the Omiecinski laboratory is currently perfrming experiments to ascertain whether this is a result of differences in protein phosphorylation or protein binding, either to the DNA or the NF-1 protein itself.

Assays of cis-acting element function have been hindered by the lack of a PB-inducible cell system that can be easily transfected with chimeric gene constructs. Nonetheless, there is a body of evidence suggesting critical elements for the CYP2B1 and 2B2 PB response lie far upstream of the transcription start site. Firstly, previous experiments in transgenic mice demonstrated the requirement for DNA sequences 5' of -800bp for CYP2B2 PB inducibility. In the absence of these sequences, CYP2B2 was expressed at high levels consitutively (41). Secondly, fragments representing CYP2B2 5' nucleotides from -2318 to -2155 partially induced downstream reporter gene expression with PB exposure in primary rat hepatocytes (60). In the study presented in Appendix B, transgenic mice experiments were performed using CYP2B2 sequences driving transcription of a CAT reporter gene. Only a few lines from each construct expressed the reporter gene, a result probably illustrating the inefficient transcription of the intronless CAT gene in whole animal systems observed by others (78). Of those with detectable CAT expression, PB-induciblity of the transgene was observed only in reporter genes driven by the full 2500bp of CYP2B2 near 5' flanking DNA. Loss of nucleotides between -2497 and -1679, which included the NF-1 site, resulted in significant levels of constitutive expression and a concomitant loss of PB-inducibility. An NF-1 element upstream of the interleukin-5 (IL-5) gene has an analogous effect on a CAT reporter gene expression in stably transfected T-lymphocytes (79). In this study, IL-5 gene 5' flanking sequence required an NF-1 binding motif in order to direct phorbol 12-myristate 13-acetate (PMA) inducible expression of the CAT gene; deletion of this element resulted in constitutive, non-inducible CAT expression. Because of studies suggesting the nucleosomal arrangement of the gene is critical for NF-1 mediated transcription enhancement (79-81), we are currently developing constructs for further analyses of the CYP2B2 NF-1 element in transgenic mice.

In this study, we present several lines of evidence that suggests NF-1 may play an important role in the regulation of CYP2B1 and CYP2B2 genes: A high-affinity NF-1 recognition element is located 2200bp 5' of the CYP2B2 gene transcription start site; The NF-1 element exists within CYP2B2 sequence required for PB-inducible gene expression in a number of systems; Although protein binds in both PB-induced and uninduced states, it binds more protein after PB exposure. The identical 34bp sequence protected in *in vitro* footprinting experiments is present in the same location in the CYP2B1 gene. Just 5' of this NF-1 element, at about -2.5kb, the 97% identity that exists between the CYP2B1 and CYP2B2 genes breaks down.

MATERIALS AND METHODS

ELECTROPHORETIC MOBILITY SHIFT ASSAY.

CYP2B2 sequence extending from -2256 to -2186 was obtained by digestion of plasmid CYP2B2 -2271/-2186pBluescript KS- with *Nco*I and *Hind*III. The 70bp fragment containing the NF-1 consensus element was radiolabeled using a Klenow fill-in reaction with α-^{32}P-dCTP, and purified from the parent plasmid on a non-denaturing polyacrylamide gel. Electrophoretic mobility shift assays (EMSA) were performed as described (58). Crude (1-5mg) or affinity purified (1µl) nuclear proteins were incubated 15 minutes at room temperature with 1mg calf thymus DNA in a buffer consisting of 10mM HEPES, 12% glycerol, 0.2mM EDTA, 5mM MgCl2, 1mM DTT, 50 µg/ml BSA, and 150mM KCl. A further 10 minute incubation followed addition of 15,000 cpm (about 0.014pmoles) of the radiolabeled probe. Samples were then electrophoresed on a 6% non-denaturing polyacrylamide gel as described (58). In some reactions, a 200-fold molar excess of unlabeled, double-stranded competitor DNA was added during the first incubation step. Competitor binding elements were synthetic double stranded oligonucleotides corresponding to either the CYP2B2 NF-1 consensus element protected in an *in vitro* DNase I footprinting assay (NF-1 wt; 5'-TTC CTG ACC TTG GCA CAG TGC CAC CAT CAA CTT G-3'), or a version of this sequence mutated at two guanine

residues critical for NF-1 binding (62,63) (NF-1 mut; 5'-TTC CTG ACC TTT GCA CAG TGC AAC CAT CAA CTT G-3'); or consensus double-stranded oligonucleotides for CTF/NF-1 (5'-CCT TTG GCA TGC TGC CAA TAT-3'; Promega), or NF-κB (5'-AGT TGA GGG GAC TTT CCC AGG C-3'; Santa Cruz Biotechnologies).

AFFINITY PURIFICATION OF DNA-BINDING PROTEIN.

Enrichment of nuclear extracts for proteins with specificity for the CYP2B2 protein binding element was based on a previously reported affinity purification method (64). A Klenow fill in reaction was performed on 400mg of *Xba*I and *Pst*I cut plasmid CYP2B2-2271/-2186pBluescript KS- in the presence of 5 nmoles biotin-16-dUTP(Enzo), followed by digestion with *Bst*XI. Coupling of the protein binding site to magnetic beads was then achieved by incubating the resulting DNA with M280-Streptavidin Dynabeads (Dynal) at a ratio of 15pmoles of insert DNA/mg Dynabeads as described (64). Affinity purification was performed separately on crude nuclear extracts prepared from whole rat livers and primary rat hepatocyte cultures, both with and without PB induction. In each experiment, identical quantities (about 1mg) of control or PB-induced nuclear extracts were incubated with 7.5mg of CYP2B2-2271/-2186 coated Dynabeads at room temperature for 30 minutes. Incubations were performed in the described TGED buffer (64) containing 150mM KCl (150mM KCl-TGED). Dynabeads were washed 4-10 times with 150mM KCL-TGED containing polydIdC or calf thymus DNA in a 10 fold excess by weight of the coupled insert DNA. In some cases, a last wash step with 400mM KCl-TGED was included. Proteins were eluted in 100 µl of 1.5M KCl-TGED, dialysed 1 hour at 4°C on .025 µm Millipore "V" Series Membranes (Millipore) against 200 ml 40mM KCl-TGED, and stored at -80°C.

PROTEIN ELECTROPHORESIS AND IMMUNOBLOTTING.

Equal volumes of affinity purified proteins from control or PB-induced nuclear extracts were separated via SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a Mini-Protean II 10% Tris-HCl precast gel (Bio-Rad) according to the manufacturer's protocol. To visualize total protein composition of the affinity-purified extracts, some gels were stained with either Coomassie blue or electroblotted onto nitrocellulose, with subsequent colloidal gold staining (Bio-Rad). Others were transferred onto Immobilon P membranes

(Millipore) for immunoblot analyses. Membranes prepared for immunoblots were each probed with a series of three antibodies; anti-NF-1, anti-phosphotyrosine-HRP (PY20), anti-phosphoserine (Zymed), and anti-NF-κB p65 (A) (Santa Cruz Biotechnology, Inc.) using conditions specified by the supplier. The secondary antibody for anti-NF-1, anti-phosphoserine and anti-NF-κB assays was goat-anti-rabbit-HRP (Santa Cruz Biotechnology Inc.) at a 1:2,000 dilution. ECL (Amersham) or SuperSignal Substrate (Pierce) was used for chemiluminescent visualization of specific immunoreactive proteins. Before re-probing, blots were stripped with a 30 minute wash in 62.5mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, and 100mM β-mercaptoethanol at 50°C.

CELL CULTURE.

Rat hepatocytes were isolated by a modification of the two-step collagenase perfusion in situ (82), and cultured with a modification of a protocol previously described (15). Briefly, cells were isolated from non-induced Sprague-Dawley rat liver and plated in Williams E medium (Gibco) supplemented with 2mM L-glutamine (Sigma), 10mM Hepes (Sigma),pH 7.4, ITS+(6.25 mg/ml transferrin, 6.25 ng/ml selenious acid, 1.25 mg/ml bovine serum albumin, 5.35 mg linoleic acid)(Collaborative), Pen/Strep (100 units/ml penicillin and 1 mg/ml streptomycin)(Sigma), 1nM dexamethasone (Sigma), and 8% NuSerum IV (Collaborative). Cells were allowed to attach for 3 hours and were then washed with EBSS (Gibco) to remove any debris and unattached cells. Fresh serum-free, fully supplemented Williams E medium was added and an overlay of Matrigel (Collaborative), 120 ug/ml final concentration, was then added. For the subsequent culturing period the dexamethasone concentration was reduced to 25nM. Fresh supplemented Williams E media was added 16 hours later. For treatments, phenobarbital was added at a concentration of 0.1mM, and di-butyryl cAMP was used at 10μM. RNA was harvested using a guanidinium isothiocyanate protocol (42).

MRNA ISOLATION AND NORTHERN BLOT ANALYSIS.

Poly A+ RNA was extracted from total RNA obtained from control and PB-induced rat livers using the PolyATtract mRNA Isolation System III (Promega). 3 mg of each sample was run on a 1% agarose, 6% formaldehyde RNA gel and blotted onto a GeneScreen

membrane according to previously reported methods (41). Membranes were hybridized to a radiolabeled cDNA corresponding to NF1-L (59). The NF1-L probe was generated in the following manner. RT-PCR as described above was performed on total RNA from PB-induced rat liver. Primer NF1-L RP (5'-GTCCAACACTGACGAATCGG-3') was used to prime the reverse transcriptase reaction and was complementary to NF1-L coding sequence at the 3' end. The PCR reaction was performed using NF1-L RP and NF1-L FP (5'-GATATCCGACCCGAGTACCG-3'), an oligonucleotide homologous to 5' NF1-L coding sequence. The appropriate-sized PCR product was purified on a 1% low-melt agarose gel and random-primed labeled with α-^{32}P-dCTP (Decaprime kit, Ambion). The signal intensity of NF1-L cDNA-RNA hybrids were quantified via densitometry and normalized to values obtained from a separate hybridization with a radiolabeled oligonucleotide complementary to albumin transcripts.

CHAPTER 4: SUMMARY AND CONCLUSIONS

The prevalent hypothesis of PB induction among researchers in the cytochrome P450 field is that a transcription activator selectively binds within the promoter of inducible genes after PB exposure. The research presented here, however, suggests a contradictory view of the mechanism of PB-induction that includes the following elements: 1. A PB-responsive element is located far upstream of the CYP2B1 and CYP2B2 gene promoters. Cis-acting DNA essential for PB-induction of a CYP2B2 transgene in mice is between -20kb and -0.8kb according to results presented in chapter 1. Recent experiments, performed by others, confirm this result and further delineate the PB-responsive portion of the CYP2B2 gene to DNA between -2318 and -2155 (60). 2. CYP2B2 is constitutively repressed by DNA 5' of -.8kb; PB induction results in a relief of this repression. In the absence of a CYP2B2 fragment from -20kb and -.8kb, the CYP2B2 transgene is expressed constitutively at levels achieved by endogenous CYP2B2 in the rat after PB treatment. Furthermore, transient CAT construct transfection assays indicate that a number of cis-acting transcriptional repressors are located upstream of the CYP2B1 and CYP2B2 promoters (chapter 2). At least two are located 5' of -802; one between -2497 and -1400, another between -1400 to -802.

Analysis of protein-DNA interactions occuring in the 5' sequence of CYP2B1 and CYP2B2 identified putative recognition motifs for known transcription factor binding sites including C/EBP, HNF-5, GRE, CACCC, and Oct-1. Protein binding occured at these sites regardless of induction status, and these sites are located in DNA functioning constitutively when upstream DNA is deleted (chapter 1, 2 and appendix B). Within DNA responsive to PB in transgenic mice (appendix B) and transiently transfected primary hepatocytes (60) is a binding site for NF-1 (chapter 3, appendix 1). NF-1 is an intriguing candidate for a PB-responsive element. Although generally thought of as a transcriptional promoter, NF-1 can act as a repressor/silencer of transcription in a number of genes such as rat liver lipoprotein lipase (83), chicken cartilage matrix protein (84), and the human polyomavirus BK enhancer (85). It is also required for inducible expression of mouse

interleukin-5 in response to concanavalin A and PMA (79) and rat peripherin in response to nerve growth factor (61). Both transcription promotion and enhancement functions of the NF-1 proteins are mediated through their proline-rich C-termainal domain via an unknown mechanism (71). Other researchers have shown that NF-1 interacts with multiple proteins within the nucleoplasm that may function as co-activators of NF-1 transcription enhancement (86). Surprisingly, a mixture of these as yet uncharacterized co-activators actually repressed basal transcription in the absence of the NF-1 activator (86). NF-1 has been postulated by others to play an important role in nucleosome positioning and rearrangement during transcriptional activation (86-88). Taken together, these data suggest NF-1 may function, at least in part, as an anti-repressor. Anti-repressors are sequence-specific DNA binding proteins that can overcome chromatin-mediated gene repression by mechanisms that have not as yet been elucidated, allowing basal transcription to proceed (89).

The strongest argument against NF-1 involvement in the PB response is the fact that, not only does it bind both in control and PB-induced states, there is also no evidence by the assays presented in this dissertation that the NF-1 protein itself is post-transcriptionally modified, at least by amino acid phosphorylation, in response to PB. Furthermore, the transcript putatively encoding the NF-1 of interest is not induced by PB. Two possibilities remain whereby NF-1 could mediate PB induction: 1. Post-translational modification other than phosphorylation, such as O-glycosylation of NF-1 (90) or ADP-ribosylation, might vary with PB exposure. 2. PB could alter interactions with or activities of an NF-1 transcriptional co-activator. This latter mechanism has been implicated in TGF-β induction of collagen α2(I) by NF-1 (88). Alevizopoulos et al. (88) identified a 14 amino acid domain in a human NF-1 (hNF1-C1 or CTF-1) that mediated this induction response. It is interesting to note that the posphorylation status of these NF-1 amino acids remained unchanged with TGF-β exposure. Instead, TGF-β served to increase the affinity of a co-activator of NF-1, histone H3, for this peptide. The rat NF1-L form that we believe is important for CYP2B1 and CYP2B2 gene regulation contains a similar domain on the basis of amino acid sequence. Nancy Beck in the Omiecinski lab is currently assaying the <17kDa protein that is serine phosphorylated and which co-elutes with affinity purified

NF-1 from control rat liver nuclei for immunoreactivity to an antibody raised against histone H3.

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APPENDIX A: IDENTIFICATION OF UPSTREAM PROTEIN BINDING ELEMENT AT -2.2KB

SURVEY OF PROTEIN BINDING DOMAINS WITHIN CYP2B1 AND CYP2B2 5'SEQUENCE.

The propensity of various portions of the CYP2B2 gene to bind nuclear protein was tested using protein filter binding assays. Initial experiments examined protein binding in a battery of gene fragments approximately 1kb in length and which represented the entire first 20kb of 5' CYP2B2 flanking sequence. After incubation with nuclear protein extracts derived from either control or PB-induced rat livers, only a DNA probe containing sequence extending from -2497 to -1529 was retained on a nitrocellulose filter. Due to the low sensitivity of this assay, this result indicated the presence of a potent protein binding site within this region (91). Both control and PB-induced nuclear proteins displayed high binding affinities for this element, although we routinely found increased binding activity by PB-induced proteins.

Further protein filter binding experiments were performed to delineate sequence elements involved in this protein binding activity. As shown in Figure 18, a variety of probes representing increasingly smaller portions of the highly retained -2497 to -1529 CYP2B2 gene fragment were constructed for use in this assay. By this method the locus of nuclear protein binding activity was narrowed to an element spanning -2229 to -2186 5' of the CYP2B2 transcription start site. In all probes where protein binding was observed, strong protein-DNA interactions occurred with nuclear proteins from both control and PB-induced rat livers; the latter, however, consistently displayed increased DNA binding.

In order to identify relevant protein binding sites of the native CYP2B1 and 2B2 genes *in vivo,* DNase I hypersensitive sites were mapped. Employing this technique, three separate assays were performed, each using a distinct restriction enzyme and gene probe. Figure 19 shows a resulting autoradiogram from an assay using *Sph*I digested DNA probed with CYP2B2 -345 to +10. The DNase I hypersensitive band at approximately -2200 was consistently present all three assays. The hypersensitive site at -1400 was not observed in the other two assays due to the location of the probes used in those cases. A substantial amount of endogenous DNase activity was present within the nuclei which resulted in

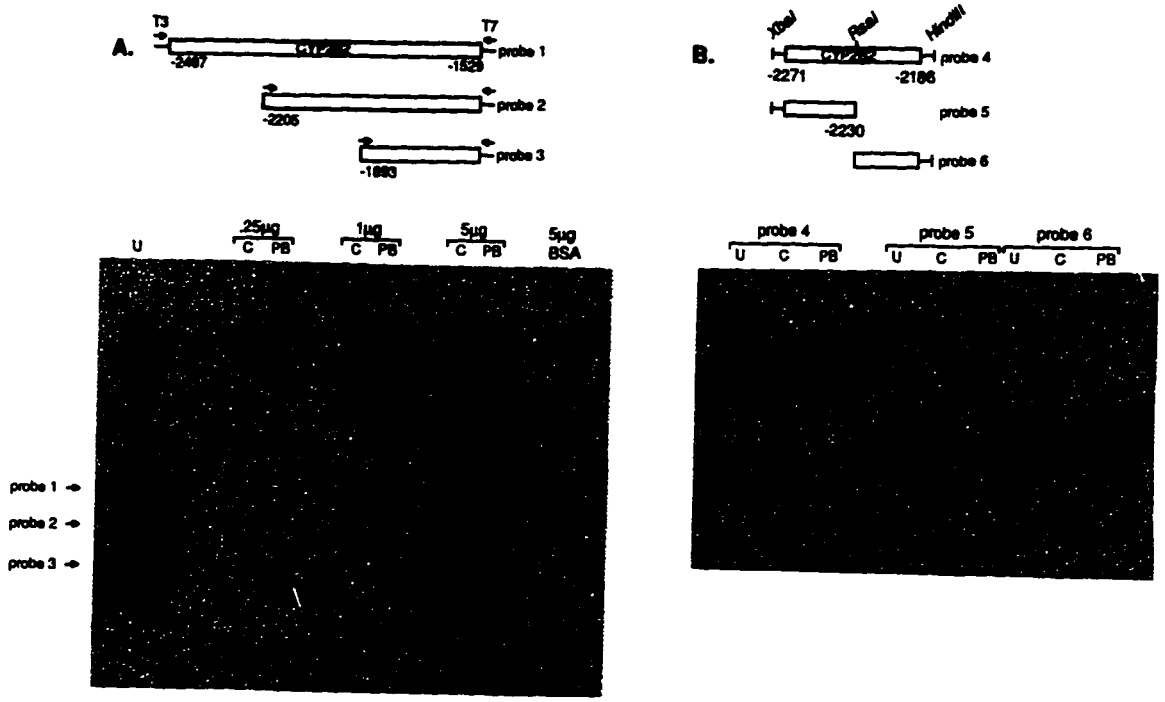

Figure 18. Identification of minimum CYP2B2 sequence retained on nitrocellulose filters by rat hepatic nuclear extracts

Panel A. Increasing amounts of control or PB-induced nuclear extracts were incubated with a mixture of radiolabeled PCR products representing various portions of CYP2B2 5' sequence from -2497 to -1529. Probes (see Materials and Methods) retained on nitrocellulose filters after vacuum blotting were liberated into a proteinase K solution, ethanol precipitated, and run on an acrylamide gel. Migration of each probe is indicated by arrows to the left of the autoradiogram. *Panel B.* Comparison of protein binding ability of different portions of the CYP2B2 5'flanking sequence from -2271 to -2186. Protein filter binding reactions were performed as above with 1ug of crude nuclear protein extracts. *U*, unfiltered probe or probe mixture; *C*, probes incubated with control nuclear protein extract; *PB*, probe(s) incubated with PB-induced nuclear protein extract; *BSA*, probes incubated with bovine serum albumin as a negative control.

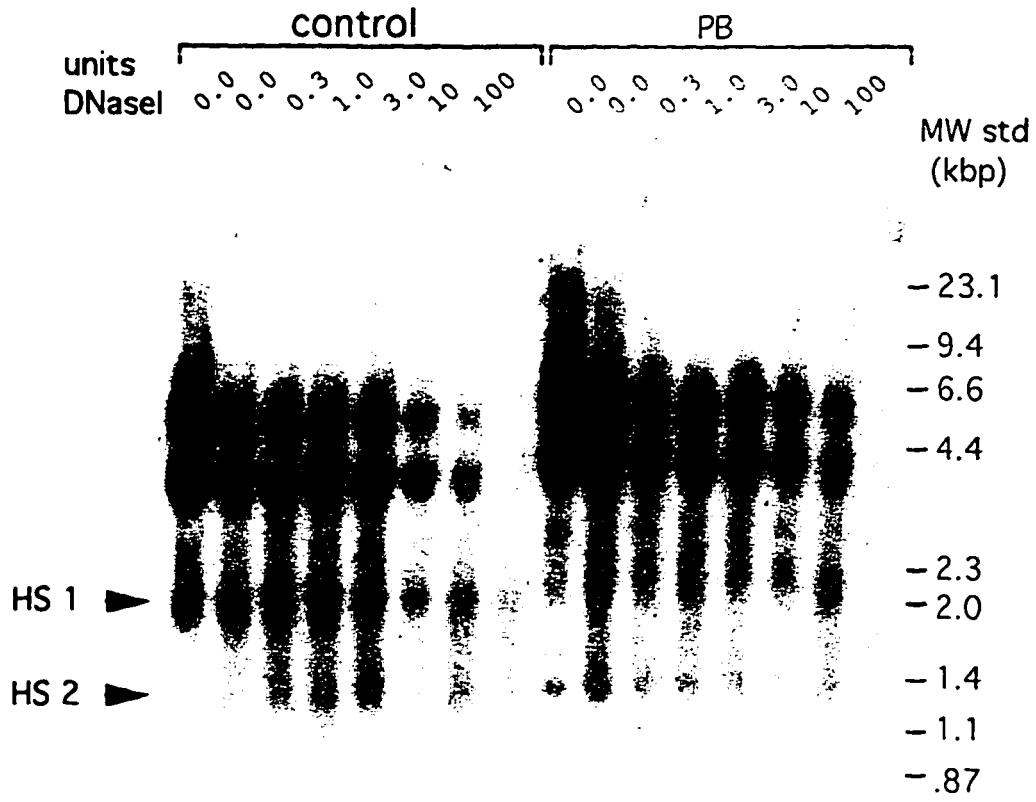

Figure 19. Detection of two DNaseI hypersensitive sites 5' of the native CYP2B1 and CYP2B2 genes.

Control or PB-induced Sprague-Dawley rat liver nuclei were incubated with increasing concentrations of DNaseI. In this figure, DNA from these nuclei were cut with SphI. The radiolabeled probe used was the CYP2B2 fragment from -345 to +10. This probe has been previously shown to be highly specific for the CYP2B1 and CYP2B2 genes (35). Migration of DNA molecular weight standards are indicated to the right. Bands at about 6 and 4kb are products of SphI digestion of the CYP2B1 and CYP2B2 genes and can be generated from restriction digest of naked DNA (data not shown). Extra low molecular weight bands (indicated by arrows) are the result of hypersensitive DNaseI cleavage at about -2.3 and -1.4kb 5' of the transcription start site.

hypersensitive bands without addition of exogenous DNaseI. These bands ar not found when DNA is extracted directly from fresh liver tissue. The size and intensity of hypersensitive bands were independent of PB-induction status.

CHARACTERIZATION OF UPSTREAM DNA BINDING ELEMENT.

In vitro DNase I footprints of a 132 bp CYP2B2 probe (-2271/-2186 CYP2B2) encompassing the high affinity protein binding site identified in the above protein filter binding and DNaseI hypersensitivity assays described above are shown in Figure 20. A single protected region was observed at the same position in both the sense and anti-sense strands. Control and PB-induced hepatic nuclear protein extracts created identical patterns of nucleotide protection. Nucleotide sequence within the protected region is similar to both NF-1 (89%) and NF-κB (67%) transcription factor binding sites, as detailed in Figure 20.

MATERIALS AND METHODS

ISOLATION OF NUCLEAR PROTEIN.

Crude nuclear protein exctracts from whole rat livers were obtained from Sprague-Dawley rats 4 or 16 hours after I.P. injection with 80mg·kg^{-1} phenobarbital in saline or with saline alone. Nuclear proteins from primary rat hepatocytes were extracted from Sprague-Dawley rat hepatocyte cells (described below) cultured 4 hours in the presence or absence of .1mM phenobarbital. Nuclear proteins were obtained as previously described (57). In some cases, cultured hepatocytes and whole livers were initially homogenized in a buffer consisting of 50mM Tris-HCl (pH7.5), 20mM KCl, 5mM $MgCl_2$, .5M sucrose, 0.15mM spermine and 0.5mM spermidine, layered over 50mM Tris-HCl (pH 7.5), 20mM KCl, 5mM $MgCl_2$, 0.88M sucrose, 0.15mM spermine, and 0.5mM spermidine, and spun at 4000 x g in an SW 28 rotor (Beckman) for 10 minutes at 4°C. Pellets were resuspended in 10mM HEPES (pH 7.6), 25mM KCl, 1mM EDTA, 2M sucrose, 19%glycerol, 0.15mM spermine, and 0.5mM spermidine, and the remaining extraction performed exactly as described (57). RNA was extracted from a portion of all samples to confirm induction.

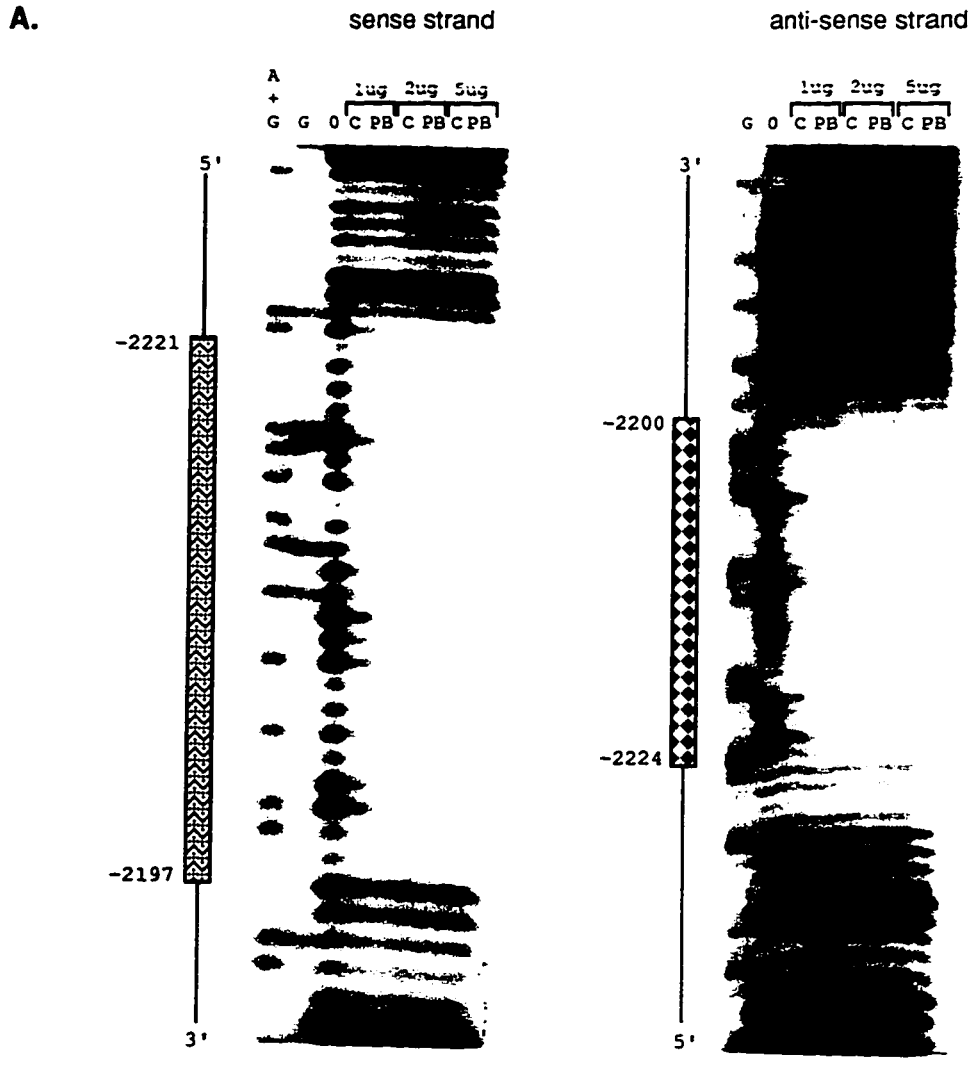

Figure 20. A single element of the CYP2B2 -2271 to -2186 gene fragment is protected from DNaseI digestion *In vitro*.

Panel A. Double stranded -2271 to -2186 CYP2B2 probes, labeled on sense or anti-sense strands, were incubated with increasing concentrations of nuclear protein as indicated. *O* = probe only; *C* = control nuclear extracts; *PB* = nuclear extracts from PB-treated rats. Position of protected bases were determined by comparison to Maxam-Gilbert *A+G* and/or *G* sequencing reactions. The region protected from DNaseI digestion by nuclear proteins is represented for each probe by a box at the left. *Panel B.* DNA sequence comparison of the CYP2B2 protected element with transcription factor recognition motifs for *NF-1* and *NF-κB* (44). Bases diverging from the CYP2B2 sequence are indicated by an asterix.

PROTEIN FILTER-BINDING ASSAY AND DNASEI HYPERSENSITIVE SITE MAPPING.

Nitrocellulose filter binding was performed as described (91). Probes used in assays depicted in figure 20 are as follows: probe 1 was generated by PCR of -2497/-1529 CYP2B2 pKS- plasmid with T3 and T7 primers in the presence of α-32P-dCTP. PCR of the same plasmid with CYP2B2 internal primers at -2205 or -1893 with the plasmid T7 reverse primer produced probes 2 and 3, respectively. A Klenow fill-in reaction with a 32-P-dCTP of *Xba*I cut -2271/-2186 CYP2B2 pKS- was followed by *Hind*III or *Rsa*I digestion to create probes 4 and 5, respectively. Probe 6 was created by first cutting the plasmid with *Hind*III, followed by Klenow labeling and *Rsa*I digestion as above. Probes were purified from the parent construct on a polyacrylamide gel using standard methods. For DNase I hypersensitive site mapping experiments, male Sprague-Dawley rats (6 week old 125g) were treated with PB at 80 mg/kg or with vehicle alone and sacrificed 16 hours later. Nuclei from liver were prepared and DNase I treated as described(92). After genomic DNA extraction, the samples were digested with restriction enzymes in the manufacturer's buffer then electrophoresed on 0.8% agarose gels, and transferred to Gene Screen Plus membrane by vacuum. Blots were hybridized as described (35). Randomly primed genomic probes were prepared as described (35).

IN VITRO DNASEI FOOTPRINTING.

A plasmid containing CYP2B2 sequence encompassing the strong protein binding activity observed upstream of the transcription start site (CYP2B2 -2271/-2186pBluescript KS-) was used to create a double stranded, single end-labeled probe for *In vitro* DNaseI footprinting experiments. *Hind*III (sense strand) or *Xba*I (anti-sense strand) digestions were followed by a Klenow fill-in reaction in the presence of α-^{32}P-dCTP. Plasmids were then digested with *Xba*I (sense strand) or *Hind*III (anti-sense strand) followed by separation of the 80bp CYP2B2 probe from pBluescript DNA by non-denaturing acrylamide gel electrophoresis. Footprinting assays were performed as described (58) with slight modifications of the binding buffer composition. 1, 2, or 5mg of crude nuclear protein extracts were incubated with 50,000 cpm of the labeled probe in 50mM NaCl, 5mM MgCl2, 1mM CaCl2, 4 μg/ml poly dIdC, 0.1mM EDTA, 20 mM HEPES (pH 7.6), 10% glycerol, 0.5mM DTT and 50 mg/ml bovine serum albumin (BSA). DNaseI digestion was carried out at a concentration of .02 units per 200ml reaction.

APPENDIX B: CYP2B2/CAT TRANSGENIC MOUSE STUDIES

EFFECT OF CYP2B2 5' SEQUENCE ON REPORTER GENE EXPRESSION IN TRANSGENIC MICE.

In a previous study of transgenic mice (41), appropriate constitutive and PB-inducible CYP2B2 expression required gene sequences between -20kb and -0.8kb 5' of the transcription start site. The aim of the present study was to test if inclusion of CYP2B2 sequence containing the high-affinity protein binding site at -2200 identified above will add back a PB-induction requirement for transcription activation to the first -.8kb of CYP2B2 5' flanking sequence. These experiments were performed using CAT in place of CYP2B2 as a reporter gene. The three chimeric CYP2B2-CAT constructs used are described in the methods. Six distinct transgenic mouse lines were established for constructs -2497/CAT and -803/CAT. Eight lines carried the -1679/CAT construct. In all lines, hepatic expression of the CAT reporter gene RNA was undetectable by enzyme or northern blot analysis regardless of induction status. CAT RNA expression could be observed with the more sensitive RT-PCR assay, but in only a subset of lines from each construct as illustrated by Figure 21. Expression patterns and quantities were independent of transgene copy number, which ranged from one to approximately 20, and were consistent for all progeny from the same lineage.

Figure 21 shows representative autoradiograms of hepatic CAT gene RT-PCR products, probed with radiolabeled CAT-specific cDNA, obtained from six individual transgenic mice lines for all three constructs. Where CAT RNA was detected, PB-inducible reporter gene expression was observed only in families transgenic for CAT constructs containing the full 2.5kb of CYP2B2 5' flanking DNA upstream. Significant levels of constitutive CAT expression, which were not modulated by PB exposure, were observed when the transgene included only the near -1.7 or -.8 kb of CYP2B2 flanking sequence

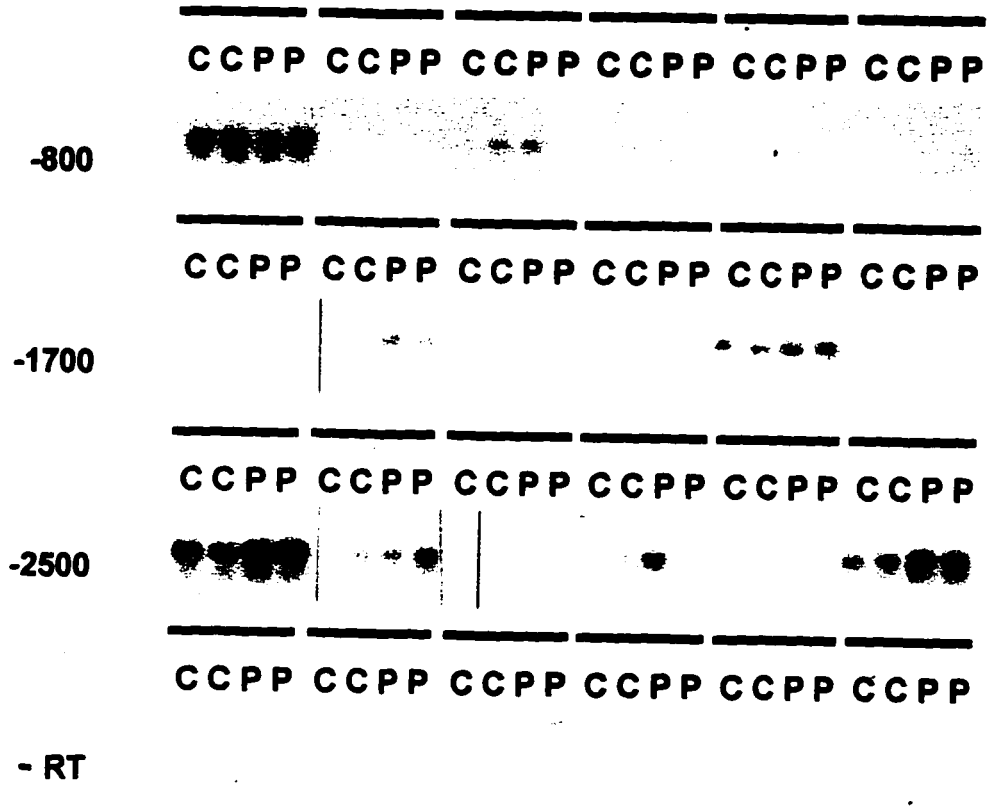

Figure 21. RT-PCR assays of liver CAT gene expression in mice transgenic for chimeric CYP2B2/CAT constructs.

Each of the first three rows presents results obtained from six distinct transgenic mice lines having the construct indicated to the left. Bars above each autoradiogram represent samples derived from the same transgenic line. For each line, assays were performed on two control animals (C) and two PB-treated animals (P). No Cat expression was detected for the two transgenic mice lines of the -1700CAT construct not presented here. To control for DNA contamination, each RT-PCR reaction was performed in duplicate, with and without reverse-transcriptase addition. Control reactions (-RT) for -2500CAT samples are presented in the last row. Identical results were obtained for the control reactions of all constructs.

MATERIALS AND METHODS

TRANSGENE CONSTRUCTIONS.

CYP2B2 flanking genomic fragments were cloned into the plasmid pBLCAT3 as previously described (41). The *BamH*I and *Bgl*II site was fused and the entire *BamH*I (CYP2B2-800)/*Sac*I (CAT cassette 3') was cloned into the corresponding sites of pBlueScript KS-, resulting in plasmid E-800/+12xCAT. The CYP2B2-CAT junction sequence was: 5'...GAT CCT GCT GGA GAG CAT GCA CTG AAG TCT Act cta gag gat ctg gat ctc gag gag ctt ggc gag att t...3'. This plasmid was digested 5' at the *Sal*I polylinker, *BamH*I at -800, treated with calf intestinal alkaline phosphatase, and ligated to the CYP2B2 genomic fragment *Xho*I (-2500)/*BamH*I (-800). The resulting plasmid, E-2500/+12 x CAT, was verified by partial sequence analysis and restriction cutting. All transgenic fragments were derived from this clone, isolated by restriction cutting (*Kpn*I: -2500/CAT; *Eco*RV + *Sac*I: -1690/CAT; *Bam*HI + *Sac*I: -800/CAT), run on low-melt agarose gels, and purified as described (41). Injections were performed by DNX.

TRANSGENE ANALYSIS.

Transgenic mice were identified and treated as previously described (41). Copy number was determined as follows. Founder DNA was digested with the restriction enzyme *Hinc*II, electrophoresed, transferred to a Gene Screen Plus membrane (NEN research products), then probed with the corresponding cloned fragment: CYP2B2 -212/CAT. Autoradiographs were taken and densitometry performed on a BioImage 3+ scanner (Millipore). Samples with faintest bands were designated as single copy to which others were standardized.

RT-PCR/PB INDUCTION.

Mice were injected with phenobarbitol at a dose of 80 mg per kilogram body weight or with vehicle alone, 0.9% NaCl, sacrificed 16-18 hr later, and RNA isolated (42). Reverse transcription was done with SuperScript II (BRL Life Tech) following Manufacturer's instructions. Eleven micrograms total RNA was pre-treated with DNase I (USB, 2units, 37°C 30min), and then annealed with a specific primer, CAT-RP (5' TGG-AAG-CCA-

TCA-CAG-ACG-GC 3'), 70°C 5min. The reaction was split, half recieving a mix of 250nM dNTPs, 10mM DTT, and 1x SuperScript buffer with 100units SuperScript (+RT) and half without the enzyme (-RT). Each 20µl reaction was incubated, 50°C 45min , then heat inactivated. Standard PCR was performed (20mM Tris-HCl, pH 8.4, 50mMKCl, 1.5mMMgcl2, 200nM dNTPs, 1.25units Taq DNA polymerase (BRL Life Tech)) on 2 µl of the RT reactions in a 50µl volume. The twenty pmol primer set used to yield a 412bp product was CAT-RP and CAT-FP (5' CTT-GCC-CGC-CTG-ATG-AAT-GC 3') then cycled (93°C 30sec, 58°C 30sec, 72°C 30sec), thirty times after as initial 2 min denaturation. One third of each sample was run on a 1% agarose gel, electrophoresed, transferred to GeneScreen membranes, then probed with a CAT cDNA probe, random p-32 labeled (Decaprime kit, Ambion).

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ABSTRACTS

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