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Transcriptional Regulation of the *CYP2B1* and *CYP2B2* Genes by C/EBP-Related Proteins

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ABSTRACT. Cytochrome P450 (CYP) 2B1 and 2B2 are encoded by two closely related genes, *CYP2B1* and *CYP2B2*, that are expressed at low levels in adult rat liver but are induced markedly by the administration of the drug phenobarbital (PB) or other structurally unrelated hydrophobic compounds to animals. Very little is understood about the molecular mechanisms that control both basal and induced transcription of these genes. We have identified two liver specific DNase I hypersensitive sites associated with the *CYP2B1* and *CYP2B2* (*CYP2B*) genes. One site, which maps to a region in the 5′-flanking region between −2.2 and −2.3 kb, became more resistant to DNase I cleavage in nuclei from PB-treated rats; the converse was true of the other hypersensitive site, which maps to the proximal promoter region between −0.05 and −0.15 kb. DNase I footprint analysis revealed three prominent and one weak footprinted regions in the promoter region in the vicinity of the proximal hypersensitive site. Using competitor oligonucleotides, we determined that one footprinted region (FT2), between −42 and −66 bp, is likely to represent a binding site for CCAATT enhancer binding protein (C/EBP) family members. Indeed, bacterial expressed recombinant C/EBPα bound at this site and formed a footprint pattern identical to the pattern observed with liver nuclear extract. *In vitro* transcription assays demonstrated that the FT2 site contributed strongly to promoter activity, since its mutation reduced transcription by 80%. Two other sites identified by footprint analysis (FT1 and FT3) are also required to maintain high basal transcription of *CYP2B2* promoter constructs in an *in vitro* transcription assay. Transient transfection experiments confirmed the expectation that C/EBPα could activate the 1.4 kb *CYP2B* promoter constructs, with mutation of the FT2 site impairing both basal transcription and transactivation by exogenous C/EBPα. BIOCHEM PHARMACOL 51;3: 345–356, 1996.

KEY WORDS. cytochrome P450; *CYP2B1*; *CYP2B2*; transcription; C/EBPα; gene; promoter

The hepatic microsomal CYPs† constitute a superfamily of monooxygenases that function in the metabolism of a wide variety of endogenous and exogenous substrates and play a central role in the detoxification of drugs and the metabolic activation of carcinogens [1–3]. Over twenty different forms of CYP with characteristic broad but overlapping substrate specificity have been detected in rat liver [4]. Many of these CYPs are interesting from a regulatory perspective since their expression involves developmental and hormonal regulation as well as tissue specificity of expression [1–3, 5].

Differential expression of the various CYPs within hepatocytes and the modulation of their levels by both endogenous (e.g. steroid hormones) and exogenous (e.g. pharmacological compounds) factors are controlled, to a great extent, at the level of gene transcription [6, 7 and reviewed recently in Ref. 5]. The hepatocyte-specific transcription of the *CYP* genes is likely to depend critically on hepatocyte-enriched transcription factors, such as HNF1, HNF3, HNF4, and C/EBP, that act in concert to sustain the transcription of other genes encoding hepatocyte-specific products [for reviews, see Refs. 8 and 9]. Some information on regulation of the *CYP* genes by these factors is now available [5].

Selective induction of the *CYP1A* subfamily members by polycyclic aromatic hydrocarbons has been examined extensively, and the molecular mechanism by which these compounds induce transcription has been characterized in some depth [reviewed in Ref. 10]. It appears that administration of polycyclic aromatic hydrocarbons leads to the dissociation of the ligand-binding AH receptor from cytosolic heat shock proteins followed by the formation of a heterodimeric transcription factor complex between the AH receptor and another polypeptide, ARNT. This complex then binds to specific *cis*-elements of the *CYP1A1* and other genes, leading to enhanced transcription. It should also be noted that proximal promoter regions in the *CYP1A1* gene play an essential role in the enhanced transcription caused by the binding of the

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† *Abbreviations:* AH, aryl hydrocarbon; ARNT, AH receptor nuclear translocator; CAT, chloramphenicol acetyl transferase; C/EBP, CCAATT enhancer binding protein; CMV, cytomegalo virus; CYP, cytochrome P450; ds, double-stranded; DSE, diad symmetry element; EMSA, electrophoretic mobility shift assay; FT, footprint; IL, interleukin; PB, phenobarbital; PCR, polymerase chain reaction; PIPES, piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]; PMSF, phenylmethylsulfonyl fluoride; SRE, serum response element; STAT, signal transducer and activator of transcription; TAE, 40 mM Tris–acetate, pH 8.0, and 1 mM EDTA; and TE, 10 mM Tris–HCl, pH 7.5, and 1 mM EDTA.

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ligand-activated AH-receptor/ARNT complex to upstream xenobiotic response elements [reviewed in Ref. 5]. This points to the importance of understanding the regulatory segments within the proximal promoter region of other inducible genes.

CYP2B1 and *CYP2B2* are the archetypal members of another subfamily of P450 genes. Their mRNA and protein levels are elevated markedly by administration of the drug PB and several other structurally unrelated hydrophobic compounds [reviewed in Ref. 11]. The induction of the *CYP2B* gene products is a consequence of enhanced gene transcription [12, 13]. A molecular mechanism for *CYP2B* gene activation by PB, however, has not been provided. Other investigators have defined several elements in the *CYP2B* genes that may contribute to either basal or PB-induced transcription [14–17]; however, nuclear proteins that bind to these promoter elements have not been identified.

In this study, using liver nuclei, we found two hypersensitive regions in the *CYP2B* genes and defined nuclear protein binding sites within one of these areas. Analysis of the proximal promoter region revealed three sites that are functionally important for transcription when promoter constructs are tested using an *in vitro* transcription assay. One element (FT2) binds recombinant C/EBPα and, therefore, may bind other transcription factors belonging to the C/EBP family. We also demonstrated, in transient transfection assays, that this site is functionally important for basal expression of a *CYP2B1* promoter construct. Moreover, in cotransfection assays, *CYP2B* promoter constructs containing a mutation at the FT2 (C/EBPα) binding site can not be transactivated by exogenous C/EBPα.

MATERIALS AND METHODS

Analysis of DNase I Hypersensitive Sites in Chromatin

Nuclei were prepared as previously described [18, 19] from control animals and those that had received an intraperitoneal injection of PB (100 mg/kg) 4 hr earlier. The nuclear pellet was resuspended in suspension buffer (15 mM Tris–HCl, pH 7.4, 150 mM NaCl, 60 mM KCl, 0.2 mM EGTA, 0.2 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, and 0.1 mM PMSF) at a DNA concentration of 0.5 mg/mL. $MgCl_2$ was added to a final concentration of 5 mM, as well as DNase I at various concentrations from 0 to 600 U/mL. The mixture was incubated on ice for 10 min, and the digestion stopped by addition of EDTA to a final concentration of 10 mM. The nuclei were collected by centrifugation, resuspended in 300 μL of suspension buffer without PMSF, and incubated with 3.5 mL of TES (50 mM Tris–HCl, pH 8.0, 2 mM EDTA, 0.1% SDS, and 0.1 mg/mL proteinase K) solution at 37° overnight with rotation/inversion. The solution was extracted with phenol, and nucleic acids were recovered by ethanol precipitation. The pellet was resuspended in 200 μL of TE and digested with DNase-free RNase for 60 min at 37°. The genomic DNA was recovered by ethanol precipitation in the presence of 2 M ammonium acetate. After digestion with the appropriate restriction endonucleases, the genomic DNA was precipitated, dissolved in TE, and subjected to agarose gel electrophoresis. Transfer of DNA to GeneScreen membranes by capillary blotting was done as recommended by the manufacturer (New England Nuclear, Boston, MA). Radioactive probes were made using the Boehringer-Mannheim (Indianapolis, IN) random priming kit method according to the instructions of the manufacturer. The specific activity of the probes was 10^9 cpm/μg. Hybridization was performed as previously described [20], except that the number of 1% SDS washes was reduced to four, and the washes were done with constant shaking in a 65° water bath.

In Vitro *Transcription Assays*

The transcriptional activity of every batch of nuclear extract was standardized by measuring the activity of 0.8 μg of DNA templates containing the adenovirus major late (pAdML) and the mouse serum albumin (pAlb320) promoter inserted into a G-free cassette (data not shown). G-free transcription assays were performed in the presence of [α-^{32}P]UTP as previously described [21].

For *in vitro* transcription assays involving *CYP* promoter templates, the method of Gorski *et al.* [21] was modified, by substituting 0.5 mM GTP for O′-6-methyl-GTP. The amount of *in vitro* synthesized, correctly initiated, labeled transcript was determined by hybridization to an appropriate antisense *CYP2B2* probe (−212 to 212 bp) that spanned the transcription start site as described [22]. To this effect, the reaction was terminated by adding 80 μL of 50 mM Tris–HCl, pH 7.4, 7 mM EDTA 0.7% SDS, 200 mg/mL of proteinase K, and 2.5 μg of unlabeled antisense −212 to 230 bp *CYP2B2* RNA probe. The solution was then incubated at 42° for 40 min and extracted with phenol, and the nucleic acid was recovered by ethanol precipitation. The purified RNA pellet was dissolved in 27.4 μL of hybridization buffer (40 mM PIPES, pH 6.4, containing 50 mM EDTA, and 80% formamide) and heated to 85°, for 10 min. After the addition of 2.4 μL of 5 M NaCl, the solution was transferred to a 55° water bath for a maximum of 16 hr. RNase digestion was performed at a concentration of 12 μg/mL RNase A. The protected transcripts were purified as described previously [22], and analyzed by denaturing polyacrylamide gel electrophoresis using 5% gels. The gels were vacuum dried and subjected to autoradiography. In quantitative assays, after autoradiography, the appropriate regions of the gel were removed and counted in a scintillation counter.

EMSA

Double-stranded oligonucleotides were prepared as described previously [23]. Incubations with nuclear extracts were performed as described for DNase I footprint analysis [23]. The DNA/protein complexes were subjected to electrophoresis on 6% polyacrylamide gels (29:1 acrylamide:bis-acrylamide) containing 5% glycerol using TAE as a running buffer at 4°. The polyacrylamide gels were dried and subjected to autoradiography as previously described [23].

Plasmid Constructions

The 1.4 kb *CYP2B1* and *CYP2B2* luciferase constructs have been described previously [24]. The 1.4 kb *CYP2B1* and

CYP2B2 CAT constructs used in transfection experiments were prepared from the luciferase constructs mentioned above [24] by isolating the *Hin*dIII-*Sph*I (approximately −1.4 kb to −5 bp) promoter fragment and inserting them into the polylinker region of pBLCAT3 [25]. Base cluster substitutions at FT2 were generated from PCR fragments as previously described [23] using oligonucleotides 1–5 listed below. All mutations were confirmed by sequence analysis. *CYP2B2* 5′ deletion mutants used for *in vitro* transcription assays and DNase I footprints were generated from a *Bam*HI-*Sac*I (−805 to 230 bp) genomic fragment cloned into pGem3 (Promega, Madison, WI). The −373 to 230 bp, the −212 to 230 bp, and the 29 to 230 bp *CYP2B2* plasmids were generated by cleavage with *Xba*I, *Hin*cII, and *Nco*I, respectively, which cut the *CYP2B2* promoter and the plasmid polylinker followed by ligation in order to circularize the plasmid. A −2.7 to −2.1 kb *Bgl*II-*Nco*I *CYP2B2* fragment was isolated after mapping a lambda Charon 4A genomic clone, λR11 [26, 27].

The proximal *CYP2B1* promoter segment used in DNase I footprinting was generated by PCR amplification from liver genomic DNA using a 5′ primer (TCCAAGATCTGCTGACAAGTGCACACCCATCCC^{-192}) that contained an artificially created *Bgl*II site (underlined) and a 3′ primer (CCGAATTCCTGACTAAGAGTAACAAGAAGCCC^{69}) whose sequence was from exon one. The amplified fragment was digested with *Bgl*II and *Nco*I, which cuts at 29 bp, and cloned into the −805 to 230 bp *CYP2B2* pGEM3 plasmid (see above) that had the *Bam*HI to *Nco*I −805 to 29 bp *CYP2B2* fragment removed.

Mutations in the *CYP2B2* promoter constructs used for *in vitro* transcription assays were made from fragments generated by PCR from the −805 to 230 bp construct and oligonucleotides 6–13 listed below, by methods previously described [23].

Synthetic Oligonucleotides Used to Generate Base Cluster Mutations in the P450 Promoter Constructs for Transient Transfection Experiments

1. *CYP2B2* upper, CGTGAACATCTGCCGCGGCCGCCTTGAGTGTAGGGGCAGATTCAGC^{-27}
2. *CYP2B1* upper, CGTGAACATCTGCCGCGGCCGCCTTGAGTGGAGGGGCGGATTCAGC^{-27}
3. *CYP2B* lower, CACTCAAGGCGGCCGCGGCAGATGTTCACGCCTCCTG^{-79}
4. Upstream *CYP2B* primer, CACATGTACCCAGGAC$^{-460\ CYP2B2}$
5. pBLCAT3 3′ primer 1, CGGTGGTATATCCAGTGA

Synthetic Oligonucleotides Used to Make Mutants for In Vitro *Transcription Assays*

6. TFT1 upper, GAGAACGCGTATAAAAGATCCTGCTGGAGAGC^{-5}
7. TFT1 lower, TTATACGCGTTCTGCCCCTACTCAGTT^{-51}
8. MFT2 upper, GTTAAGATCTTGAGTGTAGGGGCAGATTC^{-30}
9. MFT2 lower, GACCAGATCTCGAGTTCAGATGTTCACGCCTCC^{-77}
10. MFT3 upper, CCTTGGATCCATGTATGGTGTGGGTAAG^{-104}
11. MFT3 lower, ACATGGATCCAAGGACACCACGAAACAG^{-145}
12. Upper *CYP2B* primer 2, CAGAGAAGCCCCAATAATC^{-429}
13. Lower *CYP2B* primer, CCGAATTCCTGACTAAGAGTAACAAGAAGCCC^{69}

Double-Stranded Oligonucleotides Used in Transcription, EMSA and Footprint Assays

Underlined sequences represent overhangs. The superscript associated with the various oligonucleotides represents the coordinate of the 3′ base with respect to the *CYP2B1* gene. The shadowed region on the oligonucleotides below represents a C/EBP binding site (see Results), and the bases in lower case represent point mutations made in the SRE sequence, which is shown for convenience.

ds FT1 CAGATTCAGCATAAAAGATCCTGCTGGAGAGCATG^{-6}

ds FT2 GATCCGTGAACATCTG**AAGTTGCATAA**CTGAGTGTAGGG^{-38}

ds FT3 GATCCGTGGTGTCCTTGCCAACATGTATGGTGTG^{-110}

ds FT4, GATCCTAAGTCCCAGTGCCCTTTTGTCCTGT^{-150}

ds C/EBP, GATCC**ATTGCGCAAT**AATTCG

SRE sequence AATTCACAGGATGTCCAT**ATTAGGACAT**CTGCGTCAGCAGG

ds DSE, GATCCAGGATGTCCAT**ATTAGGACAT**CTGCGG

ds PR2, AATTCACAGGATGTCCAT**ATTAGGACAAac**TGCGTCAGCAGG

ds PR3, AATTCACAGGATGTCCAT**ATTAGGAgt**TCTGCGTCAGCAGG

ds KO-FAP, AATTCACAGGATGTCCAT**ATTAGGAC**ATCTGCGTgtGCAGG

The G-free cassette plasmids p(C2AT)19, pML(C2AT)19, and pAdML were gifts from Dr. R. G. Roeder (Rockefeller University, New York). The plasmids pAlb400 and PAlb320 were supplied by Dr. Uli Schibler (University of Geneva, Switzerland). We are grateful to Drs. A. D. Friedman and S. L. McKnight for the gift of *Escherichia coli* produced C/EBP protein and to Drs. R. Metz and E. Ziff (NYU Medical Center, New York) for donating the ds DSE, C/EBP, PR2, PR3, and KO-FAP oligonucleotides. The C/EBP, DSE and KO-FAP oligonucleotides are known to bind C/EBPα and β; PR2 and PR3 contain point mutations in the C/EBP binding site of the DSE oligonucleotide and do not bind C/EBP family members [23]. The C/EBPα and C/EBPβ expression vectors used in cotransfection assays with *CYP2B* CAT constructs were gifts from Dr. S. L. McKnight and Dr. U. Schibler, respectively. The C/EBPα and C/EBPβ expression vectors, cloned into pcDNA1

(Invitrogen, San Diego, CA), used in cotransfection assays with the *CYP2B* luciferase constructs were gifts from Dr. D. Ron (NYU Medical Center). All of the transcription factor expression vectors used coded for rat proteins.

Preparation of nuclear extracts, DNase I footprint analysis, cell culture, and transfection experiments were performed as described previously [24, 25]. Oligonucleotides were prepared as described previously [23]. All chemicals and reagents were molecular biology grade.

RESULTS

The sequences of the *CYP2B* genes are extremely similar up to about −2.3 kb, at which point they diverge significantly (P. M. Shaw, unpublished results). To identify possible transcriptional regulatory regions of the *CYP2B* genes, we performed DNase I hypersensitivity analysis using an intronic probe that hybridized to both genes (as indicated in Fig. 1A). Figure 1B shows that, in liver but not in brain nuclei, two regions were hypersensitive to DNase I digestion. These two regions, which map to −0.05 to −0.15 kb and −2.2 to −2.3 kb, are designated HS1 and HS2, respectively. The appearance of hypersensitive sites in liver nuclei and not brain nuclei correlates with the observed tissue specific transcription of the *CYP2B* genes [reviewed in Ref. 5]. It was also noteworthy that the intensity of the bands at HS1 and HS2 differed between control and PB-treated animals. The intensity at HS1 increased upon PB treatment, whereas the converse was true at HS2.

To identify transcription factor binding sites at the HS1 and 2 regions, we carried out footprint analysis using liver nuclear extracts. Three well-defined footprints could be detected in the proximal promoter region in the vicinity of HS1 (see Fig. 2A); however, we did not obtain consistent footprint patterns with an upstream −2.7 to −2.1 kb *CYP2B1* fragment encompassing HS2 (data not shown). The protected regions within the proximal promoter segment were observed with both of the *CYP2B* promoters and are designated FT1 (−8 to −36 bp), FT2 (−45 to −61 bp), and FT3 (−116 to −129 bp), as shown in Fig. 2A. A fourth footprint, designated FT4 (−150 to −170 bp), was also observed; however, its occupancy was variable when different preparations of nuclear extract were used. We were able to recognize the FT2 footprint as a binding site for C/EBP family members by a fortuitous set of observations. We had noticed that the region just upstream of the FT2 site shared sequence similarity to the SRE of the c-*fos* gene. Although we did not observe a footprint in this region on the *CYP2B* promoters, we found that the ds DSE oligonucleotide, which is contained within the SRE from the c-*fos* promoter, was able to inhibit markedly promoter activity in an *in vitro* transcription assay (see below). Since the SRE element is known to be capable of binding several nuclear factors including C/EBPβ [29], we determined whether it competed for the binding of nuclear factors to any of the footprints in the proximal promoter. Each of the footprints (FT1–4) formed on the *CYP2B1* promoter was specifically competed by their respective ds oligonucleotide. In addition, the ds DSE oligonucleotide could specifically compete for the FT2 footprint, whereas ds oligonucleotides that contained base substitutions in the C/EBP recognition site within the DSE sequence (oligonucleotides PR2 and PR3) could not (Fig. 3). The FT2 footprint of the *CYP2B2* promoter was eliminated by inclusion of an authentic ds C/EBP and a wild-type SRE oligonucleotide [29] in the incubation (see lanes 15–17 in Fig. 3). The competition pattern observed with this set of oligonucleotides indicated that a C/EBP-related protein was binding at FT2 and is consistent with data previously described for a C/EBP binding site in the SRE element of the c-*fos* promoter [24, 29]. We have summarized the footprint data for the *CYP2B2* gene in Fig. 4. The capacity of the ds DSE oligonucleotide to compete for complexes that bound to the FT2 region was investigated further using an EMSA. We observed that several complexes could be formed with the ds FT2 oligonucleotide and, as shown in Fig. 5A, these complexes were competed effectively by both the homologous FT2 oligonucleotide and the ds DSE oligonucleotide, and partially by the ds FT3 oligonucleotide. The ds DSE oligonucleotide was not quite as effective at competing for the formation of FT2 complexes as was the homologous ds FT2 oligonucleotide (Fig. 5B).

In summary, several footprints have been defined in the proximal promoter regions of both the *CYP2B* genes, of which the site designated FT2 appears to bind members of the C/EBP family of transcription factors. To our disappointment, we were unable to detect any reproducible differences in the footprint patterns or intensities using nuclear extracts from either control or PB-treated animals.

C/EBP is a term used to describe a family of at least six transcription factors (C/EBPα–γ, CRP1 and CHOP) [30], each of which contains a basic:leucine zipper (bZIP) motif that allows the molecules to dimerize with each other. Dimerization is a prerequisite for DNA binding and function as transcriptional regulators. The basic regions in these proteins are highly conserved and are responsible for the similar if not identical DNA binding specificities exhibited by this family of transcription factors. These proteins are known to be important in the regulation of transcription of many liver specific genes [30].

To establish definitively if C/EBP family members could bind to the FT2 region, we performed a footprint assay using both a liver nuclear extract and a purified recombinant C-terminal polypeptide segment of C/EBPα, which contains its DNA binding domain. As shown in Fig. 6, the recombinant protein generated a footprint pattern at the FT2 region on both the upper and lower DNA strands identical to that obtained with a liver nuclear extract. The footprint generated by the recombinant protein was competed effectively by both an authentic ds C/EBP and the FT2 oligonucleotide. These results demonstrate that C/EBPα can bind to the FT2 site in the *CYP2B* promoter *in vitro* and suggest that C/EBPα may play some functional role in *CYP2B* gene expression *in vivo*. It should also be noted that, as observed in the EMSA, the ds FT3 oligonucleotide was able to compete partially for the complex formed at FT2 even when that complex contained recombinant C/EBPα. This indicates that C/EBP complexes

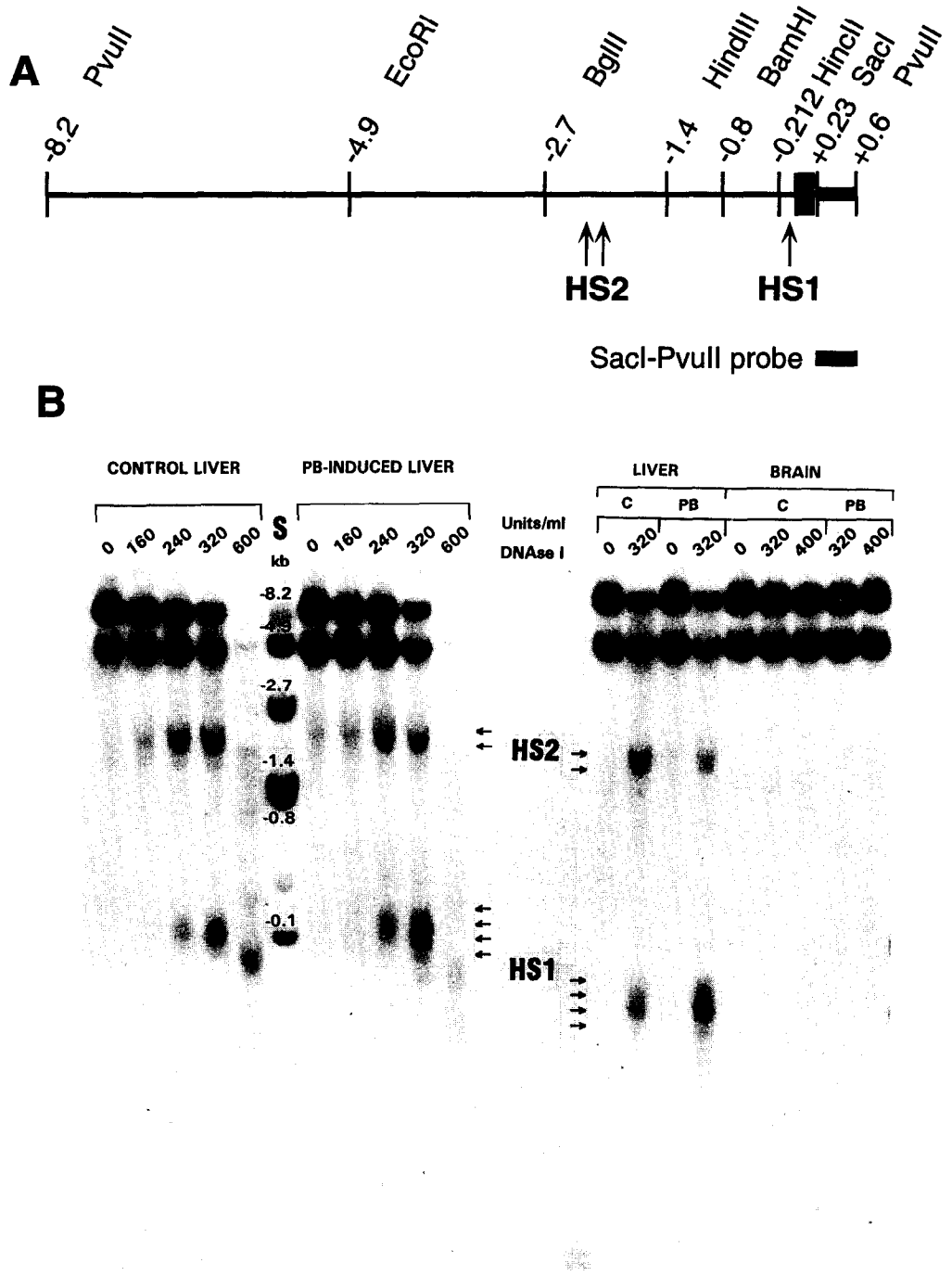


FIG. 1. Mapping of DNase I hypersensitive regions in the *CYP2B* genes. Panel A: a diagrammatic representation of a map of the *CYP2B2* gene, obtained by restriction endonuclease mapping of a genomic clone λR11 [27, 28] and by genomic Southern blotting using the SacI-*Pvu*II intronic segment as a probe. The distance from the start site of transcription of several restriction endonuclease cleavage sites is indicated. The hypersensitive sites HS1 and HS2 are indicated with arrows; exon 1 is indicated as a thick vertical bar and the SacI-*Pvu*II (0.23 to 0.6 kb) genomic fragment used to probe the Southern blots is also shown as a thick horizontal bar. This intronic fragment will hybridize to both *CYP2B* genes, due to their extensive homology over this region. Panel B: nuclei from liver and brain of control and PB-treated rats were incubated with increasing concentrations of DNase I as indicated. The DNA was extracted and processed as described in Materials and Methods and digested with *Pvu*II. The prominent band at 8.1 kb results from the *Pvu*II fragment of the *CYP2B2* gene as illustrated in panel A, and the prominent band at 4.9 kb results from a *Pvu*II fragment from the *CYP2B1* gene, which contains a *Pvu*II site around −4.3 kb. Small arrows denote bands derived from DNase I cleavage indicated as (HS). DNA size standards (S) were prepared from a mixture of genomic *CYP2B2* DNA samples that had been digested with *Pvu*II and *Eco*RI (band −4.9 kb), *Pvu*II and *Bgl*II (band −2.7 kb), *Pvu*II and *Hin*dIII (band −1.4 kb), *Pvu*II and *Bam*HI (band −0.8 kb), or *Pvu*II and *Hin*cII (band −0.1 kb). As a result, the marker fragments were detected on the Southern blot by the same indirect end-labeling strategy. The results presented were reproduced in six experiments with different preparations of nuclei. Note that the actual coordinate of the *Hin*cII site is −0.212 kb in the genomic map (panel A), but due to the presence of another *Hin*cII site at 0.5 kb (not shown), the detected band corresponds to position −0.1 kb, as indicated. The 1.4 and 0.8 kb molecular weight standards were not well resolved, resulting in a single band on the autoradiogram.

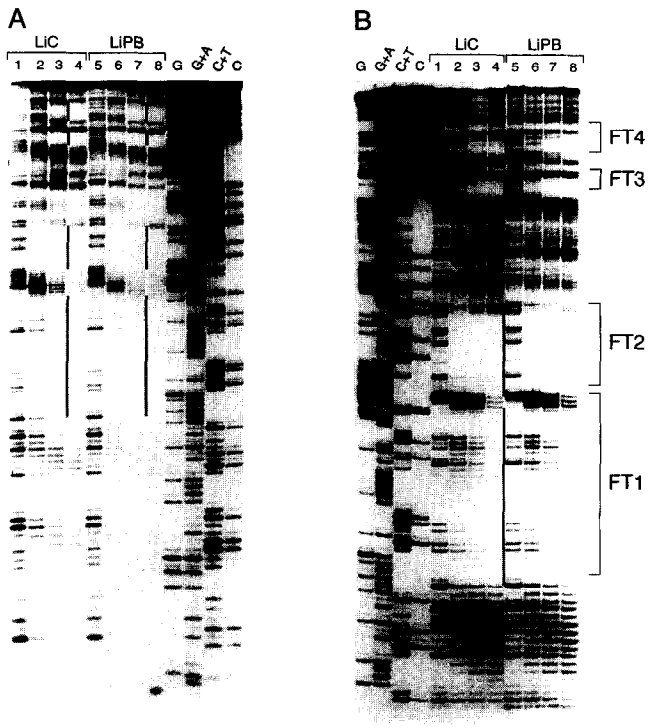


FIG. 2. DNase I footprint analysis of the *CYP2B* promoters in the vicinity of HS1. Panel A: DNase I footprint analysis of the *CYP2B2* fragment from −212 to 29 bp was performed as previously described [25]. Lanes 1 through 4 correspond to DNA samples incubated with 0, 3, 30 and 60 μg of liver nuclear extract prepared from untreated animals (LiC) before DNase I digestion. Lanes 5 through 8 correspond to equivalent reactions with liver nuclear extract prepared from PB-treated animals (LiPB). Footprinted regions FT1 through 4 are indicated with vertical lines outside and within the autoradiogram. The Maxam and Gilbert sequencing reactions G, G + A, C, and C + T of the *CYP2B2* promoter segment were run in parallel in order to identify the sequence within the footprinted areas. In panel B, a similar experiment was carried out using the *CYP2B1* promoter fragment. The amount of protein used in lanes 1–4 and 5–8 was 0, 6.5, 13 and 26 μg, respectively.

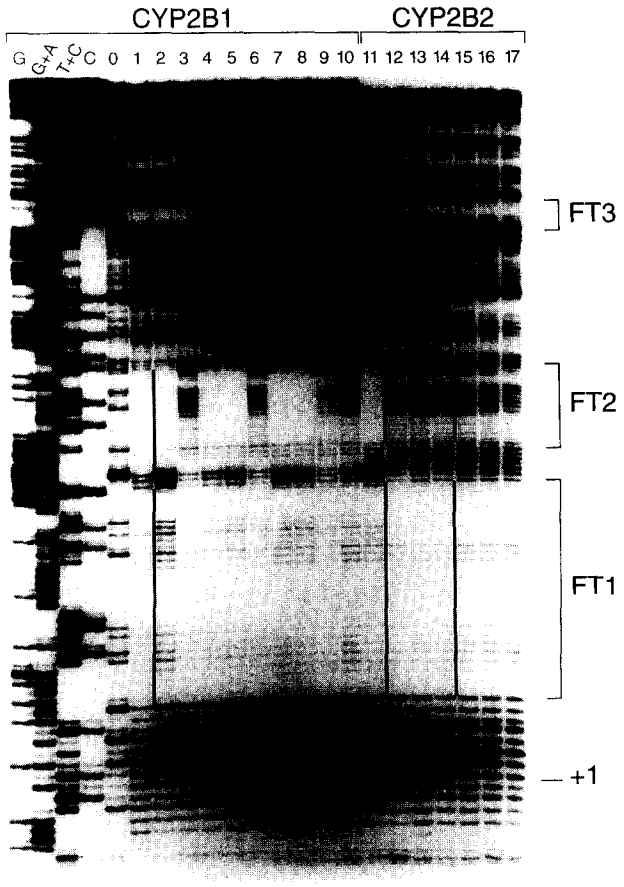


FIG. 3. Binding of C/EBP-related proteins at the FT2 site. Footprint analysis was preformed as described in the legend to Fig. 2. (Lanes 1–10) the *CYP2B1* promoter segment (−212 to 29 bp) incubated with 26 μg of liver nuclear extract from untreated animals in the presence and absence of a 100-fold molar excess of ds competitor oligonucleotides: lane 0, no nuclear extract; Lane 1, no competitor; lanes 2–10, ds FT1, FT2, FT3, FT4, DSE, PR3, PR2, KO-FAP, and C/EBP, respectively. (Lanes 11 through 17) the *CYP2B2* promoter in the presence of 26 μg of liver nuclear extract from untreated animals and an increasing molar excess of ds competitor oligonucleotides: lane 11, no competitor; lanes 12–14 correspond to a 100-, 200-, and 300-fold molar excess of ds FT2 oligonucleotide; lanes 15–17 correspond to a 100-, 200-, and 300-fold molar excess of ds C/EBP oligonucleotide. The Maxam and Gilbert sequencing reactions G, G + A, C, and C + T of the *CYP2B1* segment were run in parallel in order to identify the sequence within the footprinted areas, the start site of transcription is indicated as +1.

may be able to bind weakly to the FT3 site even though the FT3 sequence resembles a CTF/NF-1 consensus binding site.

To determine if the FT2 region played a functional role in *CYP2B* transcription, we performed transient transfection experiments in the highly differentiated FGC4 hepatoma cell line [31] using a wild-type and mutant construct of a 1.4 kb *CYP2B1* promoter. The results shown in Fig. 7 indicate that the *CYP2B1* promoter is expressed at a low but measurable level in FGC4 cells and that the eight bp substitution at FT2 in the mutant construct caused a marked decrease in promoter activity. This result suggests that the FT2 site plays an important role in transcription in these differentiated hepatoma cells. Next, we performed cotransfection assays in human C33 bladder carcinoma [32] and HepG2 cells [33] to test the *CYP2B* constructs for their ability to be activated by two transcription factors, C/EBPα and C/EBPβ, that could bind to the FT2 site. The rationale for using C33 and HepG2 cells was that these cell lines do not express the *CYP2B* promoter constructs at high levels; therefore, the effect of cotransfected C/EBP should be more marked on the *CYP2B* promoter constructs. The results from these experiments, shown in Fig. 8, demonstrate that the wild-type constructs could be activated (10- to 29-fold) by C/EBPα, but more importantly, that transactivation of the FT2 mutant constructs was considerably lower. The wild-type constructs were activated to a somewhat lesser extent by C/EBPβ. Together these results prove that C/EBPα and C/EBPβ can play a functional role in modulating transcription of both of the *CYP2B* promoters *in vitro*.

We also used an *in vitro* transcription assay and ds competitor oligonucleotides to test for functional activity of the foot-

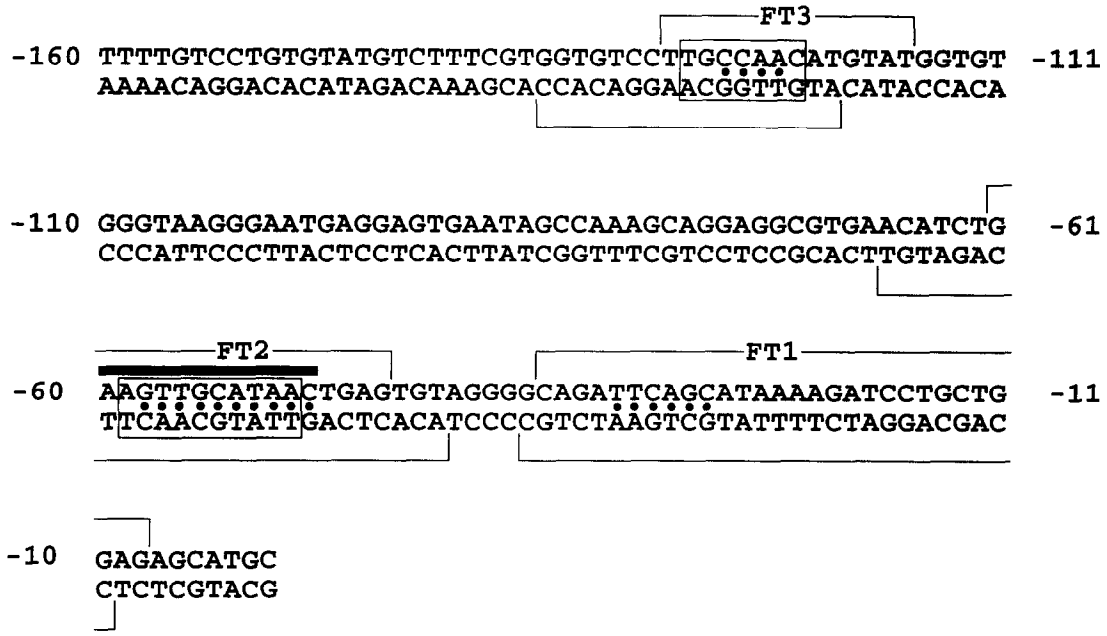


FIG. 4. Summary of the footprint data on the *CYP2B2* promoter. The extent of protection for footprints FT1–3 with liver nuclear extract is shown on the sequence of the upper and lower strands of the *CYP2B2* gene with brackets. Also indicated by dots in between the strands are the regions where mutations were made for *in vitro* transcription studies (see text); the nucleotides replaced were: at FT1, ACGCGT; at FT2, CTCGAGATCT; at FT3, GATC. The putative C/EBP and CTF/NF-1 binding sites at FT2 and FT3, respectively, are shown as boxed regions. Also shown with a horizontal bar is the region that was mutated at FT2 in the constructs used for the transfection assays; the wild-type sequence was replaced with CCGCGGCCGCCT.

printed regions within the proximal promoter of *CYP2B2*. Transcription from the −212 to 230 bp construct was decreased markedly when competitor oligonucleotide spanning regions FT1, FT2, and FT3 were included in the assay (Fig. 9), indicating that the nuclear factors that bound to these regions were important for transcription. The loss of transcriptional activity when the ds FT3 oligonucleotide was included in the assay may result from competition for proteins binding to both the FT3 and FT2 region, because, as shown above, FT3 can compete for complexes that bind to the FT2 sequence. The loss of activity caused by inclusion of the ds FT1 oligonucleotide is not surprising, as this region spans the putative TATA motif. The ds DSE oligonucleotide, which, as shown above, can compete for nuclear proteins that bind at FT2, was also an effective competitor in the transcription assay, again demonstrating the functional importance of C/EBP proteins in transcription of the *CYP2B* promoter. We also made six bp substitutions at the FT2 and FT3 sites and tested these constructs in the *in vitro* transcription assay. The experiment, depicted in Fig. 10, indicates that mutations at FT2 and FT3 reduce transcription from the *CYP2B2* promoter, with the FT2 mutation having a substantially greater deleterious effect than that of FT3. The fact that the sum of the loss of transcriptional activity when FT2 and FT3 are mutated individually is greater than 100% may indicate that the factors that bind to these sites cooperate with other factors or regions within the proximal promoter to enhance transcription from the *CYP2B2* promoter.

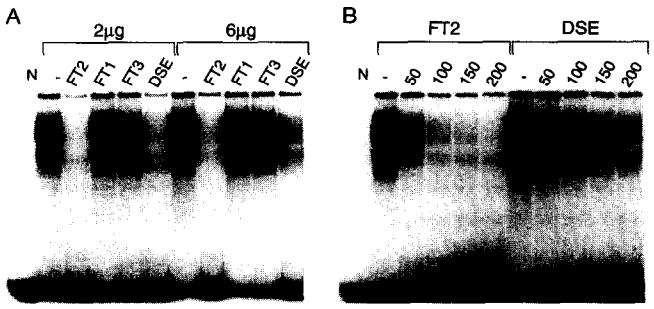


FIG. 5. Competition by the DSE element of the c-*fos* promoter for the binding of rat liver nuclear proteins to the FT2 sequence. The EMSAs were performed as described in Materials and Methods. Panel A: Radiolabeled FT2 oligonucleotide was incubated with liver nuclear extract from untreated animals as indicated. Double-stranded unlabeled competitor ds oligonucleotides used at 100-fold molar excess were included in the incubations as indicated. N corresponds to no protein. Panel B: the assay was performed as described above with 6 μg of liver nuclear extract from untreated animals in the presence of increasing molar concentrations of either ds FT2 or ds DSE competitor oligonucleotide, as indicated.

DISCUSSION

Several lines of evidence suggest that the molecular mechanism by which PB enhances transcription from the *CYP2B* genes could be quite complex, and may involve multiple *cis*-

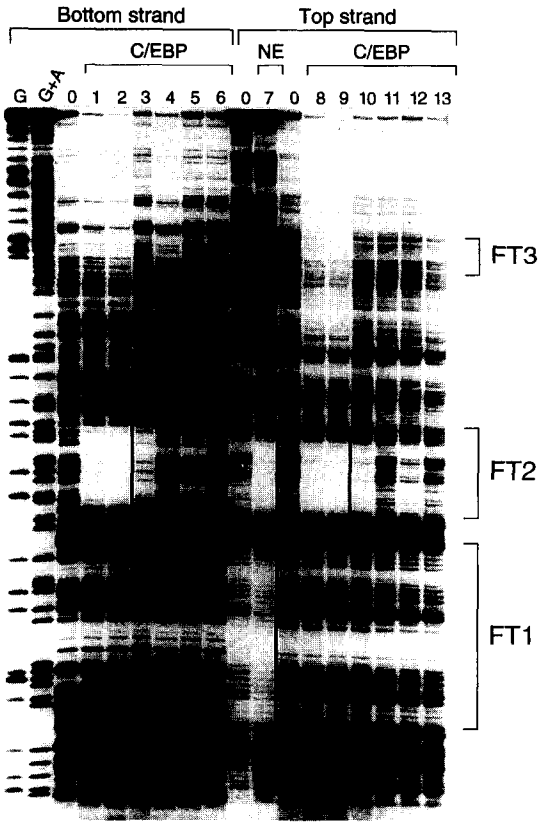


FIG. 6. DNase I footprinting analysis of the binding of recombinant C/EBPα to the FT2 site. Footprint analysis was performed as described in the legend of Fig. 2. Footprint patterns on the bottom and top strands of the *CYP2B2* promoter fragment are as indicated. Lane 0, no extract; lanes 1 and 8, 2.5 ng of recombinant C/EBPα; lanes 2 and 9, 10 ng of recombinant C/EBPα; lanes 3–6 and 10–13, 2.5 ng of recombinant C/EBPα and a 200-fold molar excess of the following ds oligonucleotides competitors: FT1 (3 and 10), FT2 (4 and 11), FT3 (5 and 12), and C/EBP (6 and 13). Lane 7 contains 10 μg liver nuclear extract (NE) from untreated animals. The footprinted regions FT1–3 are indicated with vertical lines outside and within the autoradiogram. The Maxam and Gilbert sequencing reactions G and G + A were run in parallel for the bottom strand only.

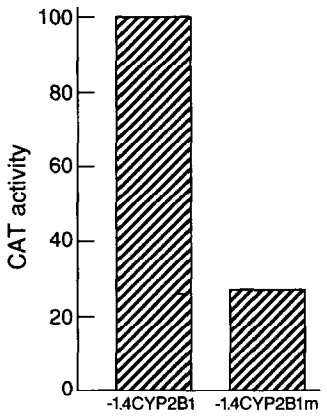


FIG. 7. Effect of mutation of the C/EBP binding site on *CYP2B1* promoter activity. Transient transfections were performed in FGC4 cells as described in Materials and Methods. To account for differences in recovery and transfection efficiency, CAT activity was normalized to β-galactosidase activity obtained with a cotransfected pCMVβ (Clontech, Palo Alto, CA) plasmid, as described previously [24]. The normalized activity of the wild-type chimeric 1.4 kb *CYP2B1* CAT construct (−1.4CYP2B1) is arbitrarily defined as 100% and represents 1.4% conversion of chloramphenicol to acetylated products. The normalized activity of the FT2 mutant construct (−1.4CYP2B1m) is given as a percentage of that of the wild-type construct. The results shown are the average of duplicate plates.

acting elements within the 5′-flanking region. Recent advances in primary culture techniques have enabled the transfection of primary hepatocytes under conditions where the endogenous CYP2B mRNAs and their translation products appear to be induced by PB treatment [34], although there has been no evidence that demonstrates that the increases observed are due to transcriptional activation. In a recent study, a 163 bp segment of the rat *CYP2B2* 5′-flanking region at approximately −2.1 kb was found to mediate PB-induced transcription from transfected *CYP2B2* promoter constructs [35]. This segment was also able to confer PB-induced transcription on a heterologous thymidine kinase promoter, although the magnitude of the induction showed experimental variation that was probably dependent on the basal activity of the thymidine kinase promoter [35]. Another study in which *CYP2B2* constructs were introduced into transgenic mice indicated that PB-responsive elements may also reside 5′ of −800 bp [36]. On the other hand, other investigators have reported increased binding of nuclear proteins from PB-treated rats to several defined regions within the proximal *CYP2B2* promoter [14, 15, 17]. However, to date, no specific transcription factors have been shown to play a role in *CYP2B* basal or induced transcription.

We observed two DNase I hypersensitive sites, HS1 and HS2, associated with the *CYP2B* genes and have focused, in this study, on the proximal promoter region in the vicinity of HS1. Although we have not defined binding sites outside the proximal 200 bp, as mentioned above, others have shown by transfection of primary hepatocytes, using a series of *CYP2B2* chimeric promoter constructs, that a 163 bp segment, which is in the vicinity of HS2, can mediate a PB response [35]. These workers also demonstrated an increased binding of liver nuclear factors from PB-treated animals using an EMSA with the 163 bp fragment. However, the factors that bind within this region remain to be characterized.

We have defined three regions within the proximal promoter of *CYP2B1* and *CYP2B2* that are protected in a DNase I footprint assay with both PB and control liver nuclear extracts. The protection pattern we observed in this region is very similar to the pattern of DNase I footprints observed by others in a recently published paper [17], except that we did not detect reproducible differences between PB and control nuclear extracts at the FT2 region, nor did we observe another footprint at −183 to −199 bp, possibly due to the length of the promoter segment that we used in our footprint assays. We cannot explain our inability to detect quantitative differences in the footprint patterns at the FT2 site using nuclear extracts

prepared from PB-treated and untreated animals, but assume that this reflects differences in the preparations of nuclear extracts. The nuclear extracts that we prepared were obtained using a protocol different from that of Shephard *et al.* [17], but they were active in supporting transcription from the albumin, adenovirus major late, and *CYP2B2* promoter constructs. In agreement with the results of Shephard *et al.* [17] we were unable to detect any footprint encompassing the region between −73 and −89 bp. This region shares sequence homology with a segment in the 5′-flanking region of PB-inducible bacterial genes and was reported by He and Fulco [15] to be bound more effectively, in an EMSA, by liver nuclear proteins from PB-treated than control rats. However, in contrast to our results and those of Shephard *et al.* [17], Rangarajan and Padmanaban [14] obtained a footprint from −87 to −56 bp, which extends from the region defined by He and Fulco [15] to halfway through FT2. These contradictory results more than likely are explained by the different methodologies used to prepare liver nuclear extracts by each group.

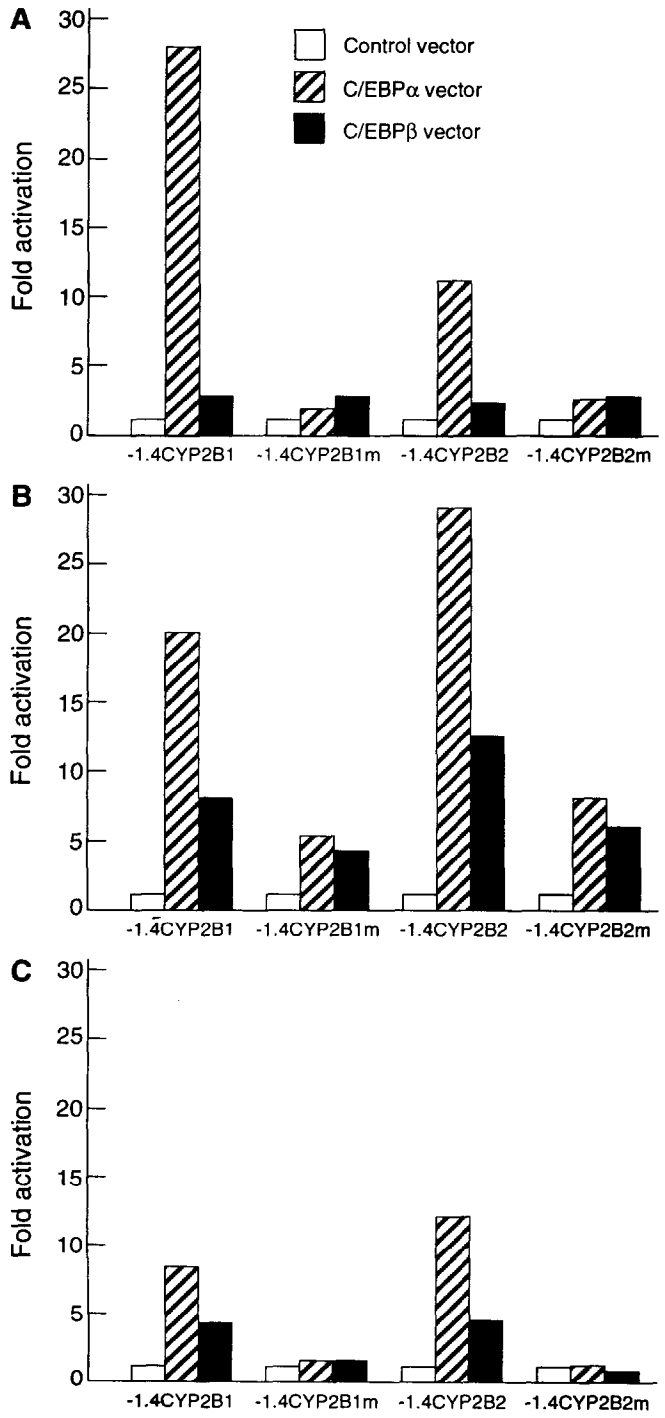


FIG. 8. Trans-activation of *CYP2B* promoter constructs with a cotransfected C/EBP expression vector. Transient transfections were performed in C33 cells with CAT (panel A) and luciferase (panel B) constructs and HepG2 cells (panel C) with luciferase constructs as described in Materials and Methods. CAT and luciferase activity were normalized as described in the legend to Fig. 7. The normalized CAT and luciferase activity of the wild-type (−1.4CYP2B) and FT2 mutant (−1.4CYP2Bm) promoter constructs in the absence of a transcription factor is arbitrarily defined as one. Cotransfections of the *CYP2B* wild-type and FT2 mutant constructs in the absence and presence of either a C/EBPα or C/EBPβ expression vector or the control vector are as indicated. The percentage of chloramphenicol converted to acetylated products, in extracts prepared from cells transfected with the −1.4CYP2B1 CAT promoter construct in the presence of the C/EBPα expression vector, ranged from 9 to 42%, in different experiments; the results shown are the averages of two experiments. The experiments performed with −1.4CYP2B luciferase constructs are the averages of duplicate plates. The actual luciferase values of the −1.4CYP2B1 construct in the presence of the C/EBPα expression vector were 7- and 66-fold higher than in the presence of the control vector pcDNA in HepG2 and C33 cells, respectively.

Of the three footprints that we observed in the proximal region of both the *CYP2B* promoters, in this paper, FT2 has been characterized the best. We have shown that the FT2 footprint pattern can be ablated by a competitor ds oligonucleotide containing a C/EBP binding site but not by ds oligonucleotides that contain base substitutions in the C/EBP binding site. In addition, the FT2 footprint observed with liver nuclear extract is essentially identical to the footprint pattern observed with a recombinant protein containing the DNA binding domain of C/EBPα. C/EBP binding sites have also been identified in two other *CYP* genes. In the *CYP2D5* gene, it was demonstrated that the binding of Sp1 facilitates binding of C/EBPβ to an adjacent weak C/EBP binding site; transfection results demonstrate a synergistic increase in transcription from the *CYP2D5* promoter in the presence of Sp1 and C/EBPβ but not C/EBPα [37]. The *CYP2C6* promoter also contains a C/EBP binding site in its proximal promoter region. In this case, however, it was shown that DBP, which also binds to that site, but not C/EBPα, can activate transcription from this promoter [24, 38].

Our transfection results together with the *in vitro* transcription data clearly show that the mutation of the FT2 site impairs transcription from *CYP2B* promoter constructs. More importantly, transactivation of the *CYP2B* promoter by C/EBPα and C/EBPβ is impaired in constructs containing a mutation at the FT2 site. Even though C/EBPα and C/EBPβ recognize identical DNA sequences, our experiments show that C/EBPα

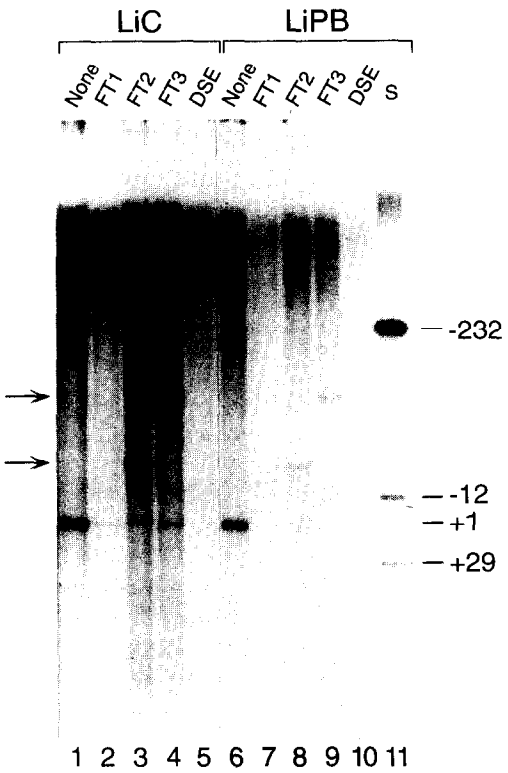
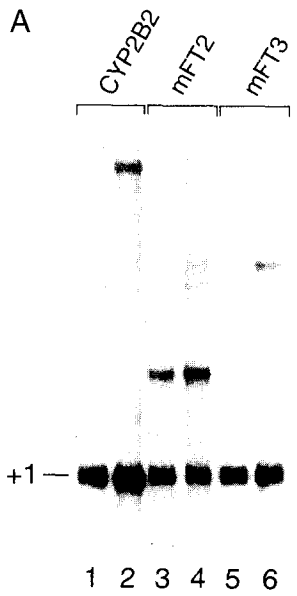


FIG. 9. Inhibition of *in vitro* transcription from the *CYP2B2* promoter by synthetic ds oligonucleotides. *CYP2B2* (−212 to 230 bp) plasmid (0.8 μg) was transcribed in the presence of 70 μg control (LiC) or PB-induced (LiPB) liver nuclear extracts and in the presence or absence of a 100-fold molar excess of the indicated ds oligonucleotide competitors. S denotes RNA size standards and +1 indicates correctly initiated transcripts. The additional reaction products in the assay, observed as a smear, represent transcripts that have started in the upstream region of the promoter construct and from RNA products that have extended more than one round along the plasmid template. The prominent bands that result when a double-stranded oligonucleotide matching the promoter template is included in the assay also represent a fraction of these transcripts. The bands are likely to be due to single-stranded oligonucleotides competing in the hybridization of the probe with the larger transcripts. This would result, after RNase digestion, in the generation of a band whose molecular weight would be the sum of 230 bp plus the distance from the start site of transcription to the region where the oligonucleotide hybridized. These bands are observed in our *in vitro* transcription assay and are shown with arrows. Note, as further evidence, that the ds DSE oligonucleotide, which is an effective FT2 competitor, does not produce this effect as it cannot hybridize to the probe or the transcripts from the promoter template. The level of the correctly initiated transcripts was not affected in the presence of 300-fold molar excess of non-specific ds oligonucleotides (data not shown).



B

Template	Percent activity	
	-212/+230	-373/+230
WT	100	100
MFT2	29	22
MFT3	53	67

FIG. 10 Effect of mutations in the *CYP2B2* promoter on its activity in *in vitro* transcription assays. Panel A: all constructs were transcribed in the presence of 30 μg of liver nuclear extract. Lanes 1 and 2 show the transcriptional activity obtained using wild-type *CYP2B2* (−212 to 230 bp) and (−373 to 230 bp) templates, respectively; lanes 3 and 4 show the transcriptional activity of the *CYP2B2* (−212 to 230 bp) and (−373 to 230 bp) templates containing a mutation at the FT2 site, respectively; lanes 5 and 6 show the transcriptional activity of the *CYP2B2* (−212 to 230 bp) and (−373 to 230 bp) templates containing a mutation at the FT3 site, respectively. +1 indicates correctly initiated transcripts. In each gel lane, the discrete band above that of the correctly initiated transcripts is derived from larger RNA transcripts as explained in the legend to Fig. 9. As the RNA segment corresponding to the mutated sequence was not protected by the wild-type antisense probe, the cleavage of this region by RNase A and RNase T_1 gave rise to a protected band of a characteristic size for each mutant sample. Panel B: the band corresponding to correctly initiated transcripts was excised from the gel and counted in a scintillation counter. The counts obtained with the wild-type templates were arbitrarily designated 100%, and the relative counts obtained with the mutant templates are shown.

increases transcription from the CYP2B promoters to a greater extent than C/EBPβ. This result is consistent with other reports in which the extent of transactivation by C/EBPα and C/EBPβ differs with the same promoter [37, 39]. It presumably reflects the context of the C/EBP binding site and complement of other nuclear factors that are present at a given time. It is interesting to note that treatment of primary hepatocytes with the cytokine IL-6, which activates C/EBPβ, inhibits the PB-mediated induction of CYP2B enzyme activity [40, 41]. IL-6 alters the transcription of several acute phase genes in liver [reviewed in Ref. 42]. The IL-6 receptor-mediated activation of acute phase genes is transduced through at least two pathways, one that involves the STAT proteins and the other through activation of C/EBPβ and possibly C/EBPδ; C/EBPα remains unchanged by IL-6 treatment [42]. One possible scenario may be that IL-6 treatment changes the occupation of the FT2 site from predominantly C/EBPα complexes to C/EBPβ complexes, thus lowering PB-mediated transcription

of the *CYP2B* genes. It appears that IL-6 activation of C/EBP factors is relatively slow, and these kinetics may be useful in determining if C/EBPβ or other C/EBP-related factors play a role in the inhibition of PB induction of *CYP2B* genes at FT2.

Our *in vitro* transcription results indicate that mutation of the FT2 and to a lesser extent the FT3 sequence reduces the transcriptional activity of the −372 and −212 bp *CYP2B2* constructs. Moreover, synthetic ds oligonucleotides containing a C/EBP binding site were able to reduce transcription of the *CYP2B2* constructs. Using a similar *in vitro* transcription assay with an extensive set of *CYP2B2* 5′ deletions, Hoffman *et al.* [16] demonstrated that a strong positive element in the proximal promoter maps to the putative PB responsive element located by He and Fulco [15] at −90 to −75 bp. We, like others [17], have not been able to detect protected regions using DNase I footprint assays within this region. Nevertheless, it is possible that these sequences are important for transcription even if proteins cannot be detected binding to these regions under the conditions we have used.

Other reported regulatory properties of the *CYP2B* genes may also be related to the role of C/EBPα in their transcription. Thus, primary hepatocytes appear to be refractory to PB induction in the first 24 hr after seeding [34, 37], which may be due to decreased C/EBPα levels caused by dissociation of the liver cells after collagenase perfusion [43]. High levels of C/EBPα are linked to the terminal differentiation of fat and liver cells [44]. Therefore, it would be interesting to determine if there is a correlation between the levels of the C/EBPα or other C/EBP-related proteins and the induction of the *CYP2B* genes by phenobarbital in primary cultures.

If, indeed, the FT2 site contributes to the PB-induced transcriptional activation of the *CYP2B* genes, then there are several different mechanisms that could affect occupancy of nuclear factors within this region. For example, (i) PB treatment may not alter the binding affinity of pre-existing transcription factors but could activate them either through phosphorylation or ligand-induced conformational change, resulting in induced transcription from the *CYP2B* genes. In this case, little or no change would be observed in this region by either footprint analysis or in an EMSA, (ii) PB may lead to an increased abundance of transcription factors that bind to the FT2 region; in this case, one should be able to observe quantitative differences between PB-treated and untreated nuclear extracts in either a DNase I footprint assay or with an EMSA. Both results (i.e. an increase and no increase in FT2 binding factors after PB treatment) have been reported in the literature, and therefore further experimentation is required to elucidate the molecular mechanisms involved by which the FT2 site may participate in PB induction.

In conclusion, we have shown that there are two sites within the proximal *CYP2B* genes that are functionally important for transcription. The FT2 site can bind C/EBP family members and can mediate transcriptional activation by C/EBPα. The FT3 site appears to match the consensus binding site of CTF/NF-1, although our results indicate that it may bind C/EBP-related proteins weakly. *In vitro* transcription studies indicate that the FT2 and the FT3 site probably cooperate with other regions within the proximal promoter to maintain basal transcription of the *CYP2B* genes. Further experiments should be performed to elucidate the cooperation of these regions in both basal and induced transcription from the *CYP2B* genes.

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