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# A model for the transcriptional regulation of the *CYP2B1/B2* gene in rat liver

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**ABSTRACT** **The phenobarbitone-responsive minimal promoter has been shown to lie between nt −179 and nt +1 in the 5′ (upstream) region of the *CYP2B1/B2* gene in rat liver, on the basis of the drug responsiveness of the sequence linked to human growth hormone gene as reporter and targeted to liver as an asialoglycoprotein–DNA complex *in vivo*. Competition analyses of the nuclear protein–DNA complexes formed in gel shift assays with the positive (nt −69 to −98) and negative (nt −126 to −160) cis elements (PE and NE, respectively) identified within this region earlier indicate that the same protein may be binding to both the elements. The protein species purified on PE and NE affinity columns appear to be identical based on SDS/PAGE analysis, where it migrates as a protein of 26–28 kDa. Traces of a high molecular weight protein (94–100 kDa) are also seen in the preparation obtained after one round of affinity chromatography. The purified protein stimulates transcription of a minigene construct containing the 179 nt on the 5′ side of the *CYP2B1/B2* gene linked to the I exon in a cell-free system from liver nuclei. The purified protein can give rise to all the three complexes (I, II, and III) with the PE, just as the crude nuclear extract, under appropriate conditions. Manipulations *in vitro* indicate that the NE has a significantly higher affinity for the dephosphorylated form than for the phosphorylated form of the protein. The PE binds both forms. Phenobarbitone treatment of the animal leads to a significant increase in the phosphorylation of the 26- to 28-kDa and 94-kDa proteins in nuclear labeling experiments followed by isolation on a PE affinity column. We propose that the protein binding predominantly to the NE in the dephosphorylated state characterizes the basal level of transcription of the *CYP2B1/B2* gene. Phenobarbitone treatment leads to phosphorylation of the protein, shifting the equilibrium toward binding to the PE. This can promote interaction with an upstream enhancer through other proteins such as the 94-kDa protein and leads to a significant activation of transcription.**

Many members of the cytochrome P-450 (*CYP*) gene superfamily are inducible. However, knowledge regarding the mechanisms of induction is restricted to a few members, although transcriptional activation is a common feature (1, 2). The mechanism of transcriptional activation of *CYP1A1* gene, induced by the prototype chemical 3-methylcholanthrene, has been studied in detail. It involves interaction of the ligand with Ah receptor, translocation of the receptor to the nucleus, and interaction with upstream cis elements (3, 4). However, the *CYP2B1/B2* gene, one of the first mammalian cytochrome P-450 genes to be identified and cloned, has presented certain unique features of regulation (5). Although phenobarbitone (PB) induces this gene (B1 and B2 are 97% homologous and hence treated as a unit at this stage) essentially through transcriptional activation, the mechanistic details are not clear. It has not yet been possible to identify a receptor for PB. The drug has pleiotropic effects in activating other genes and leads to hypertrophy of liver. Certain chemicals unrelated to PB in structure can also activate the *CYP2B1/B2* gene. One of the major problems has been the inability to activate this gene in cell culture. This poses a limitation in identifying cis-acting elements and trans-acting factors by using the conventional transfection assays in cell lines. Recent studies (6) have indicated conditions for inducing the gene in primary culture, but details regarding the kinetics of induction in relation to *in vivo* responses of the gene and exploitation of the system for identifying the regulatory features are awaited.

Studies in this laboratory have resorted to approaches based on transcription of minigene constructs of the *CYP2B2* gene in whole nuclei, run-on and cell-free transcription, DNA–protein interaction using gel shift assays, footprint analysis, and Southwestern blot analysis.

These studies have led to the identification of the near upstream region of the *CYP2B2* gene (nt +1 to −179) that contains the minimal PB-responsive promoter. This region encompasses the positive (nt −69 to −98) and negative (nt −126 to −160) cis elements (PE and NE, respectively) (7–9). Reports from two other laboratories have corroborated the PB responsiveness of the region within the PE identified in this laboratory (10, 11). However, Ramsden *et al.* (12) studying the expression of rat *CYP2B2* gene in transgenic mice have suggested that the PB-responsive element lies as far as −20 kb from the transcription start site and that such an element does not seem to be present within 800 nt upstream of the gene.

In the present study, by using DNA–asialoglycoprotein complexes to target the DNA to the liver after injection into the whole animal, we show that the near upstream region of the *CYP2B1/B2* gene (nt +1 to −179) does encompass the PB-responsive minimal promoter. The protein factor binding to the PE and NE has been purified in a functionally active form and its interaction with the cis elements has been shown to be governed by its phosphorylation status as influenced by PB administration. A model is proposed for the modulation of the PB-responsive minimal promoter based on these regulatory interactions at the PE and NE.

## MATERIALS AND METHODS

**DNA Fragments and Probes.** Plasmid pP450e179, the same as plasmid pP450e4 reported earlier (7), containing 360 nt of the *CYP2B2* gene from nt −179 to +181, was used as the template in transcription experiments. pOGH plasmid, a pUC12-based vector containing human growth hormone structural sequences inserted adjacent to a polylinker (13), was purchased from Nichols Institute (San Juan Capistrano, CA). pRSV-CAT (14) was used as an internal control in *in vivo* experiments. The plasmid containing the promoter region of phospho*enol*pyruvate carboxykinase (*Pepck*) gene of rat liver (nt −490 to +73) (15) was obtained from R. W. Hanson.

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Abbreviations: PB, phenobarbitone; PE, positive cis element; NE, negative cis element; CAT, chloramphenicol acetyltransferase.

The following oligonucleotides were synthesized by using Gene Assembler plus (Pharmacia): PE, 5′-GAGGAGTGAATAGC-CAAAGCAGGAGGCGTG-3′ (nt −98 to −69 of the *CYP2B2* gene and its complementary sequence); NE, 5′-TTTTGTCCT-GTGTATCTGTTTCGTGGTGTCCTTG-3′ (nt −160 to −127 of the *CYP2B2* gene and its complementary sequence).

**DNA Targeting and Expression in Liver.** The DNA fragment containing 179 nt of the upstream region of the *CYP2B2* gene was released with *Bam*HI–*Nco* I from pP450e179 DNA and then ligated into *Bam*HI-digested pOGH DNA, a pUC12-based vector containing human growth hormone structural sequence inserted adjacent to a polylinker (13). After ligating the *Bam*HI ends, the *Nco* I end of the insert and *Bam*HI end of the vector were filled-in and blunt-end-ligated. The plasmid is referred to as pP450GH and the DNA (50 μg) was complexed with a polylysine–asialoglycoprotein conjugate as described (16). The DNA–protein complex was injected intravenously in two doses (each of 50 μg/0.5 ml) at 24-hr interval into rats (75–80 g). In some experiments, pP450GH (50 μg) and pRSV-CAT (50 μg) were injected together as asialoglycoprotein complexes. PB (8 mg/100 g) was injected 1 hr after each injection of conjugate. The animals were killed 12 hr after the second injection of PB, and RNA was isolated from total liver and analyzed by slot blot analysis with the growth hormone DNA probe prepared from pOGH DNA. Animals receiving pOGH DNA or saline with or without PB treatment served as controls.

**Purification of the Transcription Factor.** The PE and NE (nt −69 to −98 and nt −126 to −160, respectively) were multimerized by self ligation. Multimers with ≈15 repeats (200 μg) were covalently linked to 10 ml of CNBr-activated Sepharose 2B as described (17). The coupling efficiency was ≈70%.

Nuclei were obtained from rat liver by using citric acid homogenization (18). The nuclei were suspended in 10 mM Tris·HCl, pH 7.5/50 mM NaCl/0.5 mM $MgCl_2$/50 mM sucrose/10 mM 2-mercaptoethanol. NaCl from a stock of 4 M was added drop by drop to 0.45 M, and the suspension was left on ice for 30 min with intermittent vortex mixing. The suspension was centrifuged in a SS34 (Sorvall) rotor at 12,000 rpm for 10 min and the supernatant was subjected to 80% $(NH_4)_2SO_4$ saturation. The precipitate was dissolved in 10 mM Tris·HCl, pH 7.6/1 mM EDTA/50 mM NaCl/10 mM 2-mercaptoethanol in 15% (vol/vol) glycerol and dialyzed against the same buffer for 4 hr with one change. The preparation was passed through a heparin-agarose column, and 0.3 M, 0.5 M, and 0.7 M NaCl eluates were collected on the basis of complex formation in gel mobility shift assays.

The oligo affinity column (1 ml) was equilibrated with Z buffer (25 mM Hepes, pH 7.6/12.5 mM $MgCl_2$/1 mM dithiothreitol/20% glycerol) containing 0.1 M KCl. The nuclear extract (directly or after heparin-agarose fractionation) was dialyzed against Z buffer containing 0.1 M KCl, combined with sonicated salmon sperm DNA (440 μg for 10 mg of nuclear protein), and allowed to stand for 10 min. The protein–DNA mixture was passed through the affinity column (15 ml/hr) and washed with 15 ml of the same buffer. Then 1.2 ml of Z buffer containing 1 M KCl was added to the column that was closed, and the resin was stirred and allowed to stand for 10 min. The protein was then eluted. For further purification, the eluate was diluted to 0.1 M KCl with Z buffer and mixed with competitor DNA, and column procedure was repeated. The eluted protein was concentrated by using a Centricon unit, separated into aliquots and stored at −70°C.

**Gel Mobility Shift Assay.** Nuclear extract (1–3 μg of protein) or the purified preparation was used for binding with end- or body-labeled PEs or NEs in the presence of poly(dI-dC), and samples were analyzed on low-ionic-strength 9% polyacrylamide gels as described (8). The effect of phosphorylation status of the purified factor on binding was examined by treating the protein preparation with calf intestinal alkaline phosphatase (0.6 unit) or protein kinase (catalytic subunit of protein kinase A from bovine heart muscle, 1 unit).

**Cell-Free Transcription.** Transcription extracts were prepared from control and PB-treated nuclei as described above. The extracts after 80% $(NH_4)_2SO_4$ precipitation and dialysis were used. The 20-μl reaction mixtures contained supercoiled DNA template (50 μg/ml) (pP450e179 or *Pepck*), transcription extract (50–100 μg/ml), 25 mM Hepes·KOH (pH 7.0), 50 mM KCl, 6 mM $MgCl_2$, 0.6 mM ATP, 0.6 mM GTP, 0.6 mM CTP, and 0.6 mM UTP, 12% glycerol, and 30 units of RNase inhibitor (RNasin). The transcription extracts prepared by this method were found to be very active, and 1–2 μg of protein per 20-μl reaction mixture was found to be adequate. Purified factor was incubated at 30°C for 45 min. The transcripts were isolated after RNase-free DNase and proteinase K treatments followed by phenol/$CHCl_3$ extraction and ethanol precipitation. The transcripts were quantified by RNase protection (19). Labeled RNA probes for the exonic sequences present in pP450e179 and *Pepck* were generated by using T7 RNA polymerase and [$\alpha$-$^{32}$P]UTP in a pGEM vector. The labeled RNA was hybridized with the cell-free transcripts, digested with RNases A and T1, analyzed on polyacrylamide/urea gels (8% gels), and autoradiographed.

**Labeling of Nuclear Proteins with [$\gamma$-$^{32}$P]ATP.** Nuclei (400 μg of DNA) isolated from uninduced or PB-induced rat liver were incubated with [$\gamma$-$^{32}$P]ATP (50 μCi; 1 Ci = 37 GBq) and other components used in run-on transcription (7, 8) in 200 μl at 30°C for 45 min. At the end of the incubation, the nuclei were pelleted and proteins were extracted with 0.45 M NaCl. Okadaic acid (2 μM) was included in the incubation and extraction buffers. The extracts (equal amounts of protein) were loaded onto the PE affinity column as described above. The column was thoroughly washed and then material was eluted with Z buffer containing 1 M KCl. After dilution and the addition of bovine serum albumin (50 μg), the protein was precipitated with 7% (wt/vol) trichloroacetic acid. The pellet was suspended in SDS buffer, dissociated, analyzed by SDS/PAGE (8% gels), and autoradiographed.

## RESULTS

In view of the difficulty of inducing *CYP2B1/B2* gene transcription in cell culture and the consequent inadequacy of the system to study the regulatory DNA elements by using reporter gene assays after cell transfection, we targeted the DNA to the liver *in vivo* and studied its expression. For this purpose, the 179-nt fragment of the 5′ upstream region of *CYP2B2* gene was inserted into the polylinker region of the plasmid pOGH containing growth hormone structural gene sequences as reporter. This plasmid, pP450GH, was injected as an asialoglycoprotein–polylysine complex that is directed to liver, since liver has an asialoglycoprotein receptor (16). This approach has been used to target and express genes in liver (20). The results presented in Fig. 1*A* indicate that expression of the reporter gene (namely, growth hormone mRNA) can be detected to a significant extent only in animals receiving pP450GH and PB, but not in any of the controls employed. To account for variations in DNA uptake between animals, experiments have also been conducted by injecting the two plasmid DNAs [namely, pP450GH (50 μg) and pRSV-CAT (50 μg)] together and assessing the expression of growth hormone and chloramphenicol acetyltransferase (CAT) mRNAs under these conditions. The results presented in Fig. 1*B* clearly indicate that PB treatment elicits a significant increase in growth hormone mRNA. CAT mRNA expression is seen in animals not receiving PB, and the drug treatment may slightly decrease expression. These results establish that the 179-nt sequence 5′ upstream of the *CYP2B1/B2* gene represents a minimal PB responsive promoter under *in vivo* conditions.

The next step was to decipher the regulatory interactions at the PEs (nt −69 to −98) and NEs (nt −127 to −160). The crude

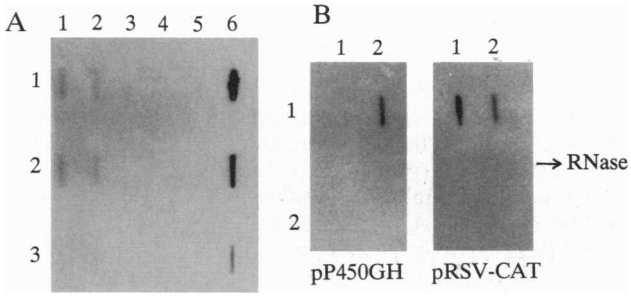


FIG. 1. Slot blot analysis of growth hormone or CAT mRNA as reporter in rat liver targeted with pP450e179 or pRSV-CAT DNA. (*A*) Rows 1–3 show signals obtained with 30, 20, and 10 μg of total RNA, respectively. The treatments were as follows. Lanes: 1, control (saline); 2, PB; 3, pOGH (plasmid DNA without promoter); 4, pOGH + PB; 5, pP450GH (plasmid DNA with 179 nt upstream); 6, pP450GH + PB. (*B*) Row 1 shows signal obtained with 20 μg of total RNA by using growth hormone or CAT probes. Row 2 shows signal after RNase treatment. The experiments have been repeated four times and the data presented are those obtained in a typical experiment. The expression of growth hormone mRNA in response to PB (lanes 2) ranges from 5- to 10-fold in various experiments over the signals obtained with saline-injected controls or nontransfected animals (lanes 1). Livers from two animals were pooled from each treatment group.

nuclear extract gives three complexes (I, II, and III) with the labeled PE. Complex III is predominantly seen at higher protein concentrations (Fig. 2). The complexes are specific and are not competed by excess of nonradioactive heterologous oligonucleotides such as SP1 and AP1. Interestingly, the complexes formed with the labeled PE are competed by an excess of PEs or NEs.

Attempts were made to purify the protein factors binding to the PEs and NEs. For this purpose, crude nuclear extracts were passed through a heparin-Sepharose column and the various salt eluates were assessed for binding to the PE in gel shift assays. The 0.3 M–0.5 M NaCl eluate gave significant binding, and this fraction was processed on the oligo affinity column prepared with multimerized PE. The affinity-purified preparation shows a sharp band at 26–28 kDa (sometimes a doublet) and very faint bands in the 90-kDa region in SDS/PAGE gels (Fig. 3). Subsequent experiments carried out with the multimerized NE have given a similar protein preparation (data not presented).

The affinity-purified preparation essentially gives rise to complex III with the PE, although faint bands corresponding to complexes I and II can also be seen. The binding of the purified preparation from the PE affinity column to the labeled PE is competed by a 10-fold excess of nonradioactive

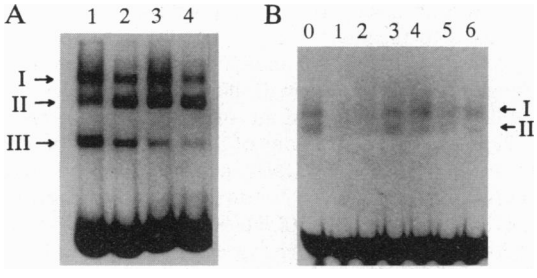


FIG. 2. Gel shift assays with labeled PE and crude nuclear extract. (*A*) Effect of protein concentration on the intensity of the complexes obtained. Lanes: 1, 2.25 μg; 2, 2.0 μg; 3, 1.75 μg; 4, 1.5 μg of protein. (*B*) Specific nature of the complexes formed. Amount of protein used was 1 μg. Nonradioactive PE and NE were used at 10-fold excess in competition experiments. The other nucleotides (SP1, AP1, and GRE) were used at 25-fold excess. Lanes: 0, no chase; 1, PE; 2, NE; 3, SP1; 4, AP1; 5, GRE; 6, GRE + AP1. Lanes 1–6 represent chase experiments with excess of unlabeled oligonucleotides. Positions of complexes I–III are indicated.

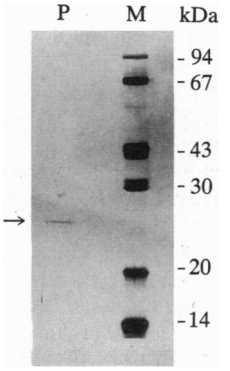


FIG. 3. Purification of protein factor on PE affinity column. The experimental details are given in the text. Briefly, 10 mg of crude nuclear extract was loaded on the heparin-agarose column and the 0.3–0.5 M NaCl eluate was dialyzed and loaded on an oligo affinity column; 25% of the 1 M salt eluate was analyzed by SDS/PAGE on 8% gels and stained with silver. Lanes: P, purified protein; M, marker proteins.

PEs as well as NEs, but not by SP1 at concentrations as high as a 50-fold excess of the labeled oligonucleotide (Fig. 4*A*). The purified preparation binds to the labeled PEs and NEs and in fact binds significantly more to the NE (Fig. 4*B*).

To examine whether modulation of the phosphorylation status of the purified preparation influences its binding to the cis elements, the preparations were treated with calf intestinal alkaline phosphatase or protein kinase and then gel shift assays were carried out. It is of interest to note that phosphatase and kinase treatment of the preparation enhance binding to the PE, the effect of kinase being more pronounced than phosphatase (Fig. 5*A*). On the other hand, kinase treatment decreases binding to the NE, whereas phosphatase treatment strikingly increases binding (Fig. 5*B*). Kinase treatment stimulates binding to the PE at higher concentrations of the purified protein when all three complexes are seen, the effect being predominantly seen with complex III (Fig. 5*C*).

To examine whether PB treatment actually leads to a change in the phosphorylation status of the factor, nuclei were isolated from uninduced and PB-induced rats. The nuclei were incubated with [$\gamma$-$^{32}$P]ATP, and the labeled protein extract was passed through the PE affinity column. Equal amounts of nuclei and extracted proteins were processed. The results presented in Fig. 6*B* clearly indicate that PB treatment leads to enhanced phosphorylation of the 26- to 28-kDa protein. In addition, enhanced phosphorylation of the 94-kDa protein is also seen. However, when the purified protein preparation is phosphorylated *in vitro* with protein kinase and [$\gamma$-$^{32}$P]ATP, the doublet is strikingly labeled (Fig. 6*A*).

The functional properties of the oligo affinity-purified preparations from PB-induced and uninduced rat liver nuclei (heparin-Sepharose step omitted) were assayed by using cell-free transcription with pP450e179 DNA as the template. In general, the transcription extract prepared from induced nu-

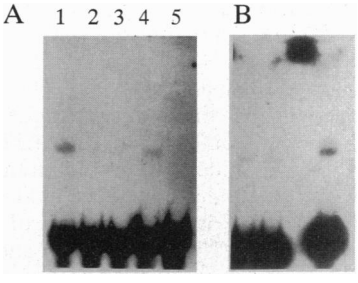


FIG. 4. Gel shift analysis with the purified factor. (*A*) The factor purified from uninduced nuclear extracts on PE affinity column was used to study binding with labeled PE. Competition carried out with a 10-fold excess of PEs or NEs and a 50-fold excess of SP1. Lanes: 1, purified factor; 2, purified factor + PE; 3, purified factor + NE; 4, purified factor + SP1; 5, no addition. (*B*) Purified factor binding to PE and NE under identical conditions. Lanes 1 and 2 are duplicates with 4 μl of purified protein fraction binding to PEs. Lanes 3 and 4 show binding to NE labeled to same specific activity as the PE with 4 μl and 1 μl of the purified protein fraction, respectively.

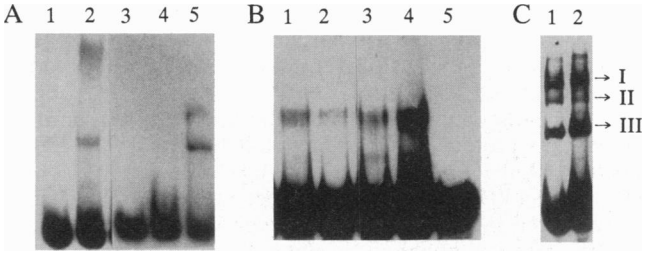


FIG. 5. (*A*) Effect of kinase and phosphatase treatment of the purified factor (4 μl) in gel shift assays with PEs. Lanes: 1 and 4, purified factor; 2, purified factor + phosphatase; 3, no additions; 5, purified factor + kinase. (*B*) NE and 1 μl of purified protein fraction. Lanes: 1 and 3, purified factor; 2, purified factor + kinase; 4, purified factor + phosphatase; 5, no addition. (*C*) PE and 12 μl of purified protein fraction. Lanes: 1, factor; 2, factor + kinase.

clei transcribes the pP450e179 DNA template used more efficiently than the uninduced extract. In the experiment presented, the PB-transcription extract is 5.2-fold more active than the control extract. The difference ranges between 2- and 7-fold with different batches of uninduced and induced cell-free extracts. The factor purified from both uninduced and induced nuclear extracts is effective in stimulating transcription, although the preparation from the induced extract is generally more effective. In the experiment presented, the factor from induced extract stimulates transcription in the cell-free transcription extract prepared from uninduced nuclei by 11.0-fold compared to the stimulation of 6.2-fold obtained with the factor purified from uninduced nuclear extract. Finally, the stimulatory effect of the purified factor is more with the uninduced cell-free transcription extract than that obtained with the preparation from PB-induced nuclei. In the experiment presented, the purified factor from uninduced and induced nuclear extracts stimulates transcription in induced extracts by 1.2- and 1.3-fold, respectively; the corresponding values for the uninduced transcription extracts are 6.2- and 11.0-fold, respectively. As stated above, the basal value from the PB-induced transcription extract is 5.2-fold more than that of the uninduced extract (Fig. 7*A*). To examine the specificity of the effects of the factor, the results obtained with pP450e179 DNA and *Pepck* DNA have been compared. Although the purified factor stimulates the transcription of pP450e179 DNA, it inhibits transcription of *Pepck* DNA (Fig. 7 *B* and *C*).

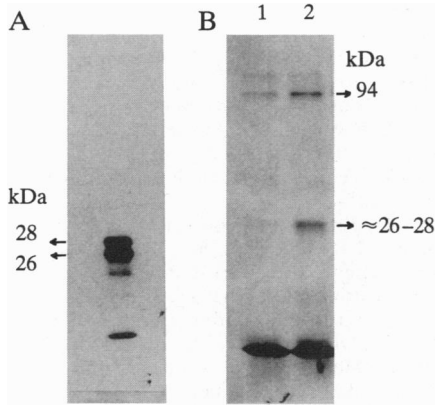


FIG. 6. Phosphorylation of protein factor in nuclei *in vitro*. (*A*) Purified factor preparation phosphorylated *in vitro* with kinase and [γ-$^{32}$P]ATP. (*B*) Control nuclei (lane 1) and PB nuclei (lane 2) phosphorylated with [γ-$^{32}$P]ATP and then factor purified on PE affinity column. Equal amounts of nuclei and extracted proteins were processed on the column. The protein preparations were analyzed by SDS/PAGE on 8% gels and autoradiographed.

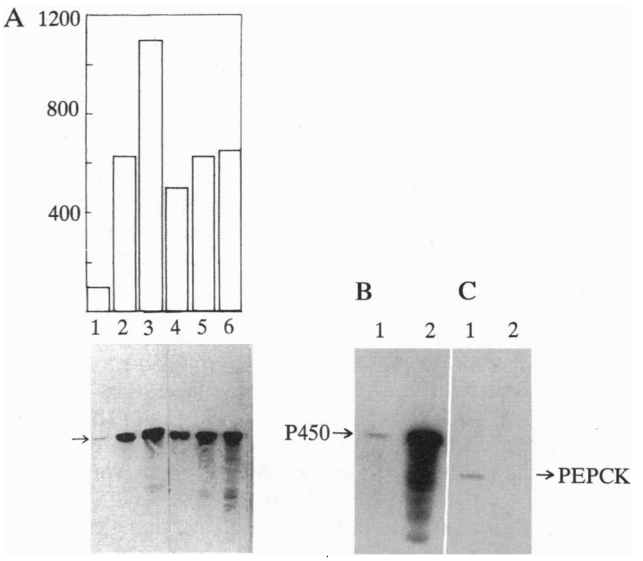


FIG. 7. (*A*) Effect of purified factor on cell-free transcription of pP450e179 DNA. Lanes and bars: 1, control cell-free transcription extract; 2, control extract + factor purified from uninduced nuclear extracts; 3, control extract + factor purified from PB-induced nuclear extracts; 4, cell-free transcription extract from PB-induced nuclei; 5, PB extract + factor purified from uninduced nuclear extracts; 6, PB extract + factor purified from PB-induced nuclear extracts. Approximately 3 ng of purified protein obtained after a single-step oligonucleotide affinity column was used. (*B*) pP450e179 DNA. Lanes: 1, control transcription extract; 2, control extract + purified protein fraction obtained after heparin agarose and oligonucleotide affinity column (2 μl). (*C*) *Pepck* DNA. Lanes: 1, control transcription extract; 2, control extract + purified protein fraction (2 μl).

## DISCUSSION

*In vitro* studies in this laboratory (7, 8) have led to the conclusion that the sequence of nt −179 to nt +1 of the 5′ upstream region of the *CYP2B2* gene harbors the minimal PB-responsive promoter. The presence of a PB-responsive element in the region from nt −69 to nt −98 has been corroborated by two other laboratories (10, 11). However, Ramsden *et al.* (12), on the basis of experiments with transgenic mice using full-length rat *CYP2B2* gene, concluded that the PB-responsive element lies as far as −20 kb and is not detectable within 800 nt of the 5′ upstream sequence. Studies with direct gene injection experiments *in vivo* presented herein have clearly revealed that there is a PB-responsive element within 179 nt of the 5′ upstream sequence of the *CYP2B2* gene. Further studies reveal that there are NEs upstream at nt −365 and downstream at nt −800 (unpublished data). This could be one reason why PB responsiveness is not observed with the entire 800-nt upstream region in transgenic experiments. In addition, a subset of the PEs identified in this laboratory (7, 8) has been referred to as a Barbie box, the PB responsive element, and identified in *Bacillus megaterium* (10), *Streptomyces griseolus* (21), and rat α-acid glycoprotein gene (22). Recently, Liang *et al.* (23) have also suggested that the results of Ramsden *et al.* (12) with transgenic mice may be open to alternate interpretations. The enhancer function of the −20-kb region is nevertheless a valid concept.

It has been possible to purify the factor(s) binding to the cis-acting elements in the near upstream of the *CYP2B2* gene. The factor is obtained as a protein of 26–28 kDa (sometimes as doublet) and is functionally active. The preparation purified on the PE affinity column stimulates cell-free transcription of pP450e179 DNA. Different batches of cell-free extracts prepared from PB-treated rat liver nuclei are 2- to 7-fold more active than control nuclear extracts in the transcription of this DNA. The purified factor from PB-treated rats in general is more effective than the preparation from control rats. Again,

the stimulation obtained with control cell-free transcription extracts is more than that obtained with PB cell-free transcription extract, the basal level being higher in the latter case. However, strict comparisons become difficult, since the protein factor yield is very low and exact quantitation of its protein content is not feasible. In addition, preliminary studies reveal that the factor may get partially dephosphorylated during bulk purification due to cellular phosphatases and the factor may also undergo phosphorylation during incubation under conditions of cell-free transcription. This is possibly the reason why purified preparations from uninduced and induced states are effective, although the preparation from induced animals is significantly more active than that from control animals in transcription extracts prepared from uninduced rats.

It is interesting to note that the same factor (26–28 kDa) in terms of mobility is purified from both PE and NE affinity columns. The factor binding to the labeled PE is competed by either the PE or NE in excess. Again, the factor purified from the PE affinity column binds to labeled PE and NE oligonucleotides. All these results indicate that the protein species binding to the PE or NE is the same. However, definitive answers would require immunological and structural identity.

Gel shift analysis with the purified factor after phosphatase and kinase treatments indicates that while the PE can bind both the phosphorylated and dephosphorylated states; the NE shows a preference for the dephosphorylated state. In fact, the factor purified from uninduced rats shows a significantly higher binding to the NE than to the PE. This laboratory has demonstrated (8, 9) that the uninduced nuclear extract binds more to the NE than does the PB-induced extract, and the reverse was found with the PE in gel shift analysis. These results and the fact that PB treatment leads to enhanced phosphorylation of the 26- to 28-kDa and 94-kDa proteins in nuclei lead us to the following proposition (Fig. 8). Under uninduced conditions, the factor (26–28 kDa) is predominantly in the dephosphorylated state and binds to the NE, keeping the gene in a repressed state, permitting only basal transcription. This interaction may involve additional protein(s), but interaction with upstream enhancer is precluded.

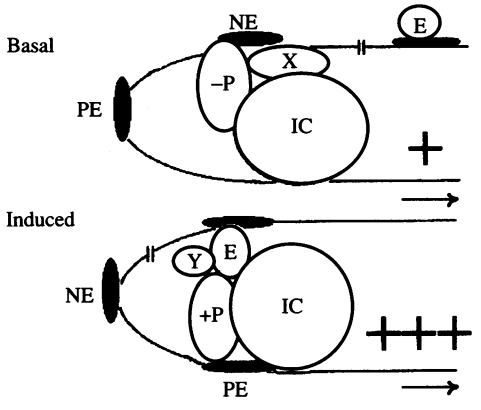


FIG. 8. Model for the transcriptional regulation of *CYP2B1/B2* gene. The basal state (+) depicts predominant interaction at the negative site (NE) through the dephosphorylated protein factor (−P) making contact with the initiation complex (IC), perhaps involving at least one other protein (X). Interaction with enhancer is precluded. The PB-induced state (+++) depicts predominant interaction of the phosphorylated protein factor (+P) with the PE, also interacting with the upstream enhancer element recognizing protein (E), perhaps involving the 94-kDa (Y) protein as well. The PE–enhancer element interaction with the initiation complex leads to the induced state of transcription. The arrow indicates the direction of transcription.

PB treatment leads to enhanced phosphorylation of the factor (26–28 kDa) shifting the equilibrium toward binding to the PE, perhaps involving additional proteins such as the 94-kDa protein that may also get phosphorylated. It is interesting to note that earlier studies from this laboratory (7, 24) identified the 94-kDa protein in nuclear extracts binding to the entire labeled 179-nt 5′ upstream sequence by Southwestern analysis. Thus, the 94-kDa protein may interact with the 26- to 28-kDa protein but is significantly lost during purification of the factor (26–28 kDa), although it is detected as a faint band in preparations obtained after a single round of affinity chromatography. As reported in this study, the purified factor in low concentrations generates essentially complex III with the PE in gel shift assays. At 5- to 10-fold higher protein concentrations, complexes I–III are generated and this may require the participation of the 94-kDa protein, the latter being present in less than stoichiometric concentrations in the purified preparation. This protein–protein interaction can promote interaction with the far upstream enhancer element, permitting striking activation of transcription by the inducer.

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