A 65-kDa Protein Mediates the Positive Role of Heme in Regulating the Transcription of CYP2B1/B2 Gene in Rat Liver*

Shahana Sultana, Chaitanya S. Nirodi, Nivedita Ram, Leena Prabhu, and Govindarajan Padmanaban‡

From the Department of Biochemistry, Indian Institute of Science, Bangalore, 560 012 India

Heme deficiency precipitated by CoCl$_2$ administration to rats leads to a striking decrease in the inducibility of CYP2B1/B2 mRNA levels and its transcription by phenobarbitone (PB), besides decreasing the basal levels. Exogenous hemin administration counteracts the effects of CoCl$_2$ administration. The binding of nuclear proteins to labeled positive cis-acting element (−69 to −98 nucleotides) in the near 5′-upstream region of the gene is inhibited by CoCl$_2$ administration to saline or PB-treated rats, as assessed in gel shift assays. Administration of exogenous hemin to the animal or addition in vitro to the extracts is able to overcome the effects of CoCl$_2$ treatment. The protein mediating this effect has been purified from CoCl$_2$ administered nuclear extracts by heparin-agarose, positive element oligonucleotide affinity, and heme affinity column chromatography. This 65-kDa protein manifests very little binding to the positive element, but in the presence of certain other nuclear proteins, shows a strong heme-responsive binding. The purified protein binds heme. It is also able to stimulate transcription of a minigene construct of the CYP2B1/B2 gene containing −179 nucleotides of the 5′-upstream region and the I exon in a cell-free system, manifesting heme response. It is concluded that the 65-kDa protein mediates the constitutive requirement of heme for the transcription of CYP2B1/B2 gene.

The transcriptional regulation of the cytochrome P-450 (CYP) supergene family is of considerable interest. The CYP1A1 gene of rat liver, induced by the prototype drug 3-methylcholanthrene, has been studied in detail. It involves interaction of the ligand, 3-methylcholanthrene, with the Ah receptor, translocation of the receptor to the nucleus, and interaction with specific upstream elements (1, 2). The details regarding the mechanism of transcriptional activation of the CYP2B1/B2 gene (2B1 and B2 are 97% homologous and hence treated as a unit) by the prototype drug, phenobarbitone (PB),[1] are beginning to emerge. Studies in this laboratory have led to the identification of a positive cis-acting element (−69 to −98 nt) and a negative cis-acting element (−126 to −160 nt) in the near 5′-upstream region of the CYP2B1/B2 gene (3–5). The positive element includes the 17-base pair PB-responsive consensus element, referred to as Barbie Box, identified first by Fulco and co-workers (6–8) in Bacillus megaterium, rat, mice, and other organisms of barbiturate inducible cytochrome P-450 and other proteins. There have also been other PB-responsive sequences identified in the CYP2B1/B2 gene of the rat. Shephard et al. (9) have identified two sequences, located between −183 to −199 nt and −31 to −72 nt, to be PB-responsive, although these do not include the "Barbie" elements. In addition, Trottier et al. (10) have located a sequence between −2155 and −2318 nt to be PB-responsive and Ramsden et al. (11) have identified an upstream enhancer as far as −20 kilobases. Studies in this laboratory have led to the development of a model which proposes that a ~26-kDa protein interacts with the positive or negative element based on its phosphorylation status. It is proposed that the binding of the ~26-kDa protein with the positive element in its phosphorylated state and with the negative element in its dephosphorylated state, within the PB-responsive minimal promoter in the near 5′-upstream region of the CYP2B1/B2 gene, would determine the inducible and basal states of the gene, respectively. The inducible state would involve the interaction of the positive element with the upstream enhancer elements, mediated through protein-protein interaction (12).

Earlier studies from this laboratory have also implicated that heme, the prosthetic group of cytochrome P-450, is a positive modulator of CYP2B1/B2 gene transcription (13–16). This conclusion is based on the fact that inhibitors of heme biosynthesis block induction of CYP2B1/B2 mRNA by PB and its run-on transcription in isolated nuclei. This inhibition is counteracted by the administration of exogenous hemin. In the present study it has been possible to demonstrate that a 65-kDa nuclear protein mediates the positive modulation of CYP2B1/B2 gene transcription by heme.

MATERIALS AND METHODS

Treatment of Animals—Rats (75–80 g) were injected with PB (8 mg/100 g, intraperitoneal), CoCl$_2$ (6 mg/100 g, subcutaneous), hemin (75 µg/100 g, intraperitoneal), or saline in appropriate combinations. CoCl$_2$ in general was injected 30 min before PB or hemin administration and the rats killed after 5 h.

Plasmids Used—The plasmid pP450e179 containing 360 nt of the CYP2B2 gene and covering positions −179 to +181 nt (3–5) was used as a template in transcription reactions. pGEM-3Z vector was used to generate riboprobes.

Gel Mobility Shift Assay—Nuclei were obtained from the livers of treated rats using the citric acid homogenization procedure (17). The nuclei were suspended in a buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM MgCl$_2$, 50 mM sucrose, and 10 mM β-mercaptoethanol. NaCl from a stock of 4 M was added drop by drop to a final concentration of 0.45 M and the suspension left on ice for 30 min with intermittent vortexing. The suspension was centrifuged at 12,000 rpm for 10 min and the supernatant dialyzed against nuclear suspension buffer.

Nuclear extract (1–3 µg) was used for binding with the body labeled positive element (5′-GAGGAGTGAATAGCCAAAGCAGGAGGCGTG-3′) covering −98 nt to −69 nt of the CYP2B2 gene annealed to a

[1] The abbreviations used are: PB, phenobarbitone; nt, nucleotide(s); PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

complementary primer and extended in presence of [α-^{32}P]dCTP. Poly(dI-dC) was used as a competitor and samples were analyzed on low ionic strength 9% polyacrylamide gels as described earlier (4, 5). A stock solution (fresh) of hemin (1 mM) was prepared in 0.1 N NaOH and then adjusted to pH 8.0 with acid. Appropriate dilutions were made with 10 mM Tris-HCl buffer (pH 8.0) using this stock for gel shift and other assays. Approximately 2 μl of hemin solution is usually added to a reaction mixture of 20 μl.

Quantification of CYP2B1/B2 mRNA—Polysomal RNA was isolated from magnesium-precipitated rat liver polysomes using phenol-chloroform extraction procedure (18). CYP2B1/B2 mRNA was quantified by RNase protection assay using [α-^{32}P]UTP-labeled I exon riboprobe. The labeled RNA probe was hybridized to 20 μg of polysomal RNA, digested with ribonuclease A and T1, and then analyzed on polyacrylamide-urea gels (8% polyacrylamide) followed by autoradiography (5).

Run-on Transcription—This was carried out by the procedure of Guertin *et al.* (19) and has been described (3, 4). Briefly, nuclei were incubated with 200 μCi of [α-^{32}P]UTP and other components including human placental RNase inhibitor in a total volume of 200 μl for 30 min at 25 °C. The labeled transcripts (10^7 cpm) were hybridized to filters containing I, VI, and IX exon of the CYP2B2 gene. The riboprobes corresponding to these exons were prepared using pGEM-3Z vector. The filters were washed inclusive of RNase treatment and then subjected to autoradiography.

Transcription in Cell-free Extracts—The nuclear extracts prepared for gel shift assays were used after 80% (NH$_4$)$_2$SO$_4$ fractionation. The precipitate was dialyzed against nuclear suspension buffer and used. The reaction in a total volume of 20 μl contained: DNA template (a minigene construct containing -179 nt of the 5′-upstream region and the I exon, pP450e179), 25 mM HEPES-KOH (pH 7.0; 50 mM KCl, 6 mM MgCl$_2$, 0.6 mM each of ATP, GTP, CTP, and UTP, glycerol (12% in final concentration), 30 units of RNase inhibitor and transcription extract (50–100 μg of protein/ml). The transcription extract prepared as described was found to be very active and 1–2 μg of protein per 20-μl reaction volume was found adequate. The reaction mixture was incubated at 30 °C for 45 min. The RNA transcripts were then isolated after RNase-free DNase and proteinase K treatments followed by phenol/CHCl$_3$ extraction and ethanol precipitation. The transcripts were quantified by the RNase protection method described earlier.

Purification of Heme Responsive Protein from Nuclear Extracts—Nuclei were prepared from CoCl$_2$-treated rats and the 0.45 M NaCl extract was dialyzed and passed through a heparin-agarose column in buffer containing 50 mM NaCl, 25 mM Tris-HCl (pH 7.4), and 2 mM EDTA. The column was washed with buffer containing 0.3 M NaCl. Further elution was carried out with buffer containing 0.4 M NaCl and then with 0.5 M NaCl. The peak protein fractions were pooled, dialyzed, and used for gel mobility shift assays. The 0.5 M NaCl eluate was further purified on oligoaffinity column. This was prepared by ligating a multimer of the positive element in pUC 19, amplified by polymerase chain reaction, to CNBr-activated Sepharose 4B (12). The column was washed with the binding buffer (25 mM HEPES-NaOH (pH 7.6), 12.5 mM MgCl$_2$, 1 mM dithiothreitol, and 20% glycerol) and eluted with buffer containing 1 M NaCl. This was loaded onto a hemin-agarose column (in the presence of 1 M NaCl), washed thoroughly, and then eluted with 2 M guanidine-HCl in 50 mM Tris-HCl (pH 8.0). The eluate was extensively dialyzed against buffer (10 mM Tris-HCl (pH 7.4), 1 mM EDTA) with repeated changes. The dialysate was concentrated using membrane filters.

Binding of 65-kDa Protein with Hemin—The purified protein preparation was analyzed on SDS-PAGE (10% gel) and proteins visualized using silver stain. To examine the heme binding property of the purified protein, the preparation was incubated with [^{59}Fe]-hemin (10 μCi/mg, kind gift from National Institute of Immunology, New Delhi) and then subjected to SDS-PAGE, with the omission of β-mercaptoethanol and the boiling step. The gel was then subjected to autoradiography. Another approach was to fractionate the protein-hemin complex on a Sepharose 4B column and identify the radioactivity peaks corresponding to protein bound and free hemin.

UV Cross-linking Analysis—This was basically carried out by pooling up quadruplicates set up for gel shift assays with the 0.5 M NaCl eluate from the heparin-agarose column and the labeled positive element. The mixture was taken in a precooled microtiter plate and irradiated for 15 min on ice with UV light (254 nm, maximum intensity: 7000 uw/cm^2) at a distance of 4 cm from the UV source. The sample was then electrophoresed on SDS-PAGE (10% gel) and autoradiographed. In another approach the gel shift assay mixture (pooled samples) after UV irradiation was analyzed by electrophoresis on a preparative 9% native polyacrylamide gel as described earlier. The wet gel was exposed to x-ray
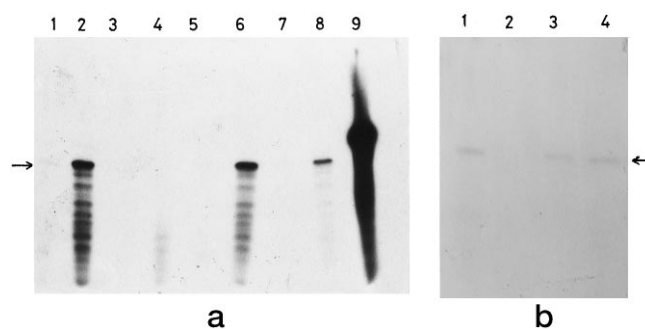

FIG. 1. **Effect of CoCl$_2$ and hemin treatments of the animal on basal and PB-induced levels of CYP2B1/B2 mRNA in rat liver.** The treatment schedule is given in text. Polysomal RNA was used to quantify CYP2B1/B2 mRNA by the RNase protection method using exon I riboprobe. *Arrow* indicates the position of the protected fragment of expected size in the autoradiogram. *a*, effect on induced levels. *Lanes 1*, control (saline); *2*, PB; *3*, CoCl$_2$; *4*, PB + CoCl$_2$; *5*, hemin; *6*, PB + hemin; *7*, CoCl$_2$ + hemin; *8*, PB + CoCl$_2$ + hemin; *9*, riboprobe. *b*, effect on basal levels. *Lanes 1*, control (saline); *2*, CoCl$_2$; *3*, hemin; *4*, CoCl$_2$ + hemin. The autoradiograms in *a* and *b* were exposed overnight and 7 days, respectively.

film overnight at 4 °C. Bands (II and III) corresponding to DNA-protein complexes were excised and once again exposed to UV. The complexes were then eluted in Tris-EDTA buffer and precipitated with 10% (w/v) trichloroacetic acid. The pellet was thoroughly washed with 70% ethanol, acetone, and ether to remove the acid. The final pellet was analyzed on SDS-PAGE as described earlier.

RESULTS

Earlier studies from this laboratory have shown that CoCl$_2$ treatment leads to a striking decrease in the heme pool (13–16). The present study has also made use of this reagent to precipitate heme deficiency. Earlier studies on mRNA quantification and run-on transcription were based on filter hybridization and measurement of radioactivity. Here these experiments have been repeated using riboprobes and the sensitive method of RNase protection assay. The results presented in Fig. 1*a* indicate that PB treatment leads to a striking increase in CYP2B1/B2 mRNA as monitored using the exon I riboprobe. CoCl$_2$ treatment leads to a striking inhibition of PB-mediated induction of CYP2B1/B2 mRNA. Exogenous hemin administration leads to a partial but significant counteraction of the effects of CoCl$_2$. Hemin administration at this concentration has no significant effects when given to saline or PB-treated rats. Administration of CoCl$_2$ to uninduced rats decreases even the basal level of CYP2B1/B2 mRNA and this can be bought back to the basal level by the administration of exogenous hemin (Fig. 1*b*). The results presented in Fig. 2 indicate that a significant increase in run-on transcription is seen after PB treatment as monitored using exon I, VI, and IX riboprobes. CoCl$_2$ treatment inhibits a PB-mediated increase in the transcription of CYP2B1/B2 gene and hemin administration is able to counteract this effect.

The results presented in Fig. 3 indicate that cell-free transcription of pP450e179, the minigene construct containing -179 nt of the 5′-upstream region and exon I, is significantly enhanced after PB treatment of the animal. The basal as well as induced levels of transcription are inhibited by CoCl$_2$ treatment. Hemin administration significantly counteracts the effects of CoCl$_2$ on PB treatment.

Studies from this laboratory have shown that the positive element (-69 to -98 nt) generates three complexes (I, II, III) with crude nuclear extract in gel shift assays and PB treatment of the animal leads to a significant intensification of the complexes (4, 12). It was, therefore, of interest to examine whether CoCl$_2$ treatment would influence binding of nuclear proteins to the positive cis-acting element. The results presented in Fig. 4
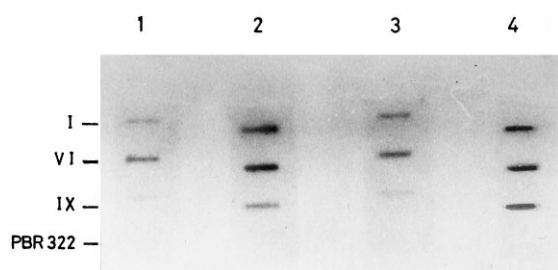
FIG. 2. **Effect of CoCl$_2$ and hemin treatments of the animal on the activation of run-on transcription of CYP2B1B2 gene by phenobarbitone in rat liver.** The experimental details are described in text. The riboprobes used are for exon I (201 nt), exon VI (300 nt), exon IX (417 nt), and pBR322. The exon I probe includes 20 nt of the polylinker region. Nuclei were prepared from animals given the following treatments: *Lanes 1*, control (saline); *2*, PB; *3*, PB + CoCl$_2$; *4*, PB + CoCl$_2$ + hemin.
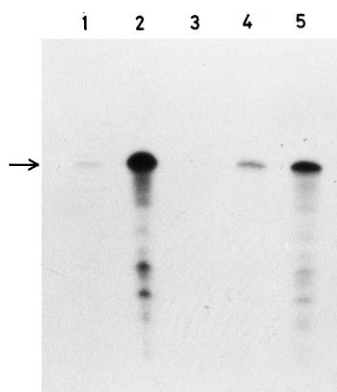

FIG. 3. **Effect of CoCl$_2$ and hemin treatments of the animal on cell-free transcription of pP450e179 DNA.** The experimental details are given in text. *Arrow* indicates the position of the protected fragment of expected size in the autoradiogram. Transcription extracts were prepared from animals given the following treatments: *Lanes 1*, control (saline); *2*, PB; *3*, CoCl$_2$; *4*, PB + CoCl$_2$; *5*, PB + CoCl$_2$ + hemin.

FIG. 4. **Effect of CoCl$_2$ and hemin treatments of the animal on the binding of nuclear proteins with the labeled positive element.** The preparation of crude nuclear extract and gel shift assay conditions are described in text. *I, II,* and *III* refer to the three complexes obtained with 1 μg of protein under the experimental conditions used. Gel shift assays were carried out in a total volume of 20 μl. The treatments given are: *lanes 1*, control (saline); *2*, CoCl$_2$; *3*, PB; *4*, PB + CoCl$_2$; *5*, hemin; *6*, PB + hemin; *7*, CoCl$_2$ + hemin; *8*, PB + CoCl$_2$ + hemin; *9*, free probe. Addition of solvent alone used to solubilize and dilute hemin at appropriate concentrations has no effect on the gel shift pattern obtained.
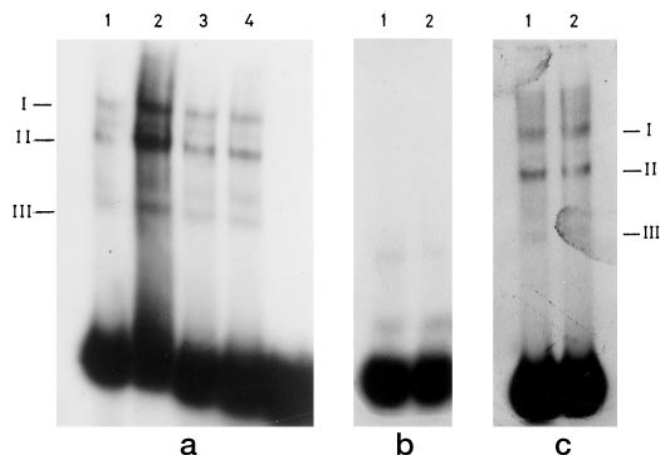
FIG. 5. **Effects of hemin, protoporphyrin, and FeCl$_3$ *in vitro* on the binding of the nuclear proteins with the labeled positive element.** The optimal hemin concentration was arrived at after testing the effect at different concentrations. *a*, nuclear extracts prepared from CoCl$_2$-treated rats were used. To clearly visualize the complexes, 2.5 μg of protein was used. *Lanes 1*, no addition; *2*, hemin (10^{-7} M); *3*, protoporphyrin (10^{-7} M); FeCl$_3$ (10^{-7} M). *b* and *c*, nuclear extracts from control animals (saline) and PB-treated animals, respectively, were used at 1 μg of protein level. *Lanes 1*, no addition; *2*, hemin (10^{-7} M).

reveal that PB treatment leads to a significant increase in the intensity of complexes I, II, and III and this increase in prevented by CoCl$_2$ treatment. Interestingly, hemin administration *in vivo* restores binding in extracts prepared from CoCl$_2$ as well as PB + CoCl$_2$-treated rats. It does not have a significant effect, when administered to saline or PB-treated rats. The results presented in Fig. 5 (*a, b,* and *c*) reveal that addition of hemin *in vitro* (maximal effect obtained at 10^{-7} M) can also stimulate binding of nuclear extracts prepared from CoCl$_2$-treated rats to the positive cis-acting element. At the level of the crude extract, all three complexes show increased binding to the positive element in the presence of heme. Again, it does not have a significant effect when added to extracts from control (saline) or PB-treated rats. The results presented in Fig. 5 also reveal that the stimulatory effect on binding is specific to hemin addition *in vitro* and is not shared by equivalent concentrations of protoporphyrin or FeCl$_3$.

The next step was to purify the heme-responsive protein factor in the nuclear extract. For this purpose, nuclear extracts prepared from CoCl$_2$-treated rats were fractionated on the heparin-agarose column. The results presented in Fig. 6 reveal that hemin addition *in vitro* specifically stimulates binding of the 0.5 M NaCl eluate from the column to the positive cis-element. Complex II shows a striking response and complex III shows a modest response. The 0.4 M NaCl eluate does not show a heme response in terms of binding. A UV cross-linking analysis of the 0.5 M NaCl eluate gives a clear complex in SDS-PAGE indicating a molecular mass of ~65 kDa after subtracting the molecular mass of the oligonucleotide used (Fig. 7*a*). UV
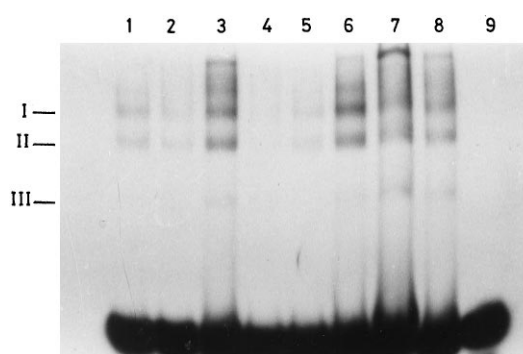
cross-linking analysis of complex II as such gives an identical pattern (data not presented). A similar analysis of complex III reveals a lower molecular mass protein in the range 23–26 kDa (Fig. 7*b*).

On this basis, the 0.5 M NaCl eluate was loaded onto a positive element affinity column and the bound proteins were eluted with 1 M NaCl. This was loaded onto the heme affinity column and the bound proteins were eluted with 2 M guanidine HCl. The eluate was extensively dialyzed and SDS-PAGE analysis reveals a single protein band of 65 kDa (Fig. 8). Next, binding studies were carried out with the purified protein and labeled positive element. The preparation shows hardly any binding to the positive element, but in the presence of small amounts of crude nuclear extract (100 ng of protein from uninduced rats), which by itself does not generate easily detectable complexes, manifests a striking heme-responsive increase in the intensity of complex II. Both the purified protein and hemin are required to elicit maximal response. Complex III shows a modest response (Fig. 9, *a* and *b*). Qualitatively similar
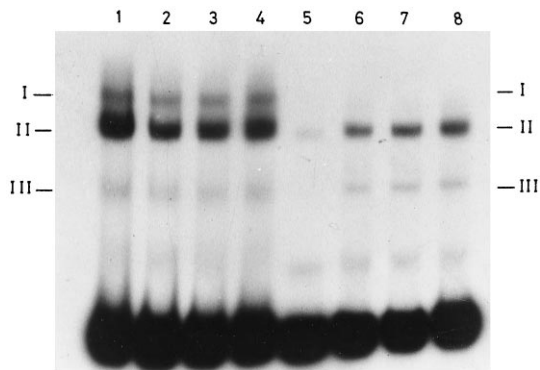
FIG. 6. **Effect of hemin *in vitro* on the binding of nuclear proteins fractionated on the heparin-agarose column with the labeled positive element.** The nuclear extract from $CoCl_2$-treated rat livers was fractionated on heparin-agarose column. The experimental details are given in the text. *Lanes 1–4* and *5–8* refer to complexes obtained with 0.4 and 0.5 M NaCl eluates from the heparin-agarose column, respectively. *Lanes 1* and *5*, no addition; *2* and *6*, hemin (10^{-7} M); *3* and *7*, hemin (10^{-8} M); *4* and *8*, hemin (10^{-9} M).

FIG. 8. **SDS-PAGE analysis of the protein purified from nuclear extract of $CoCl_2$-treated rats.** The various purification steps are given in text. The final preparation obtained after hemin-affinity column fractionation was analyzed on SDS-PAGE (10% gel) and stained with silver nitrate. *Lanes 1*, molecular weight markers; *2*, purified protein: *ov*, ovalbumin.
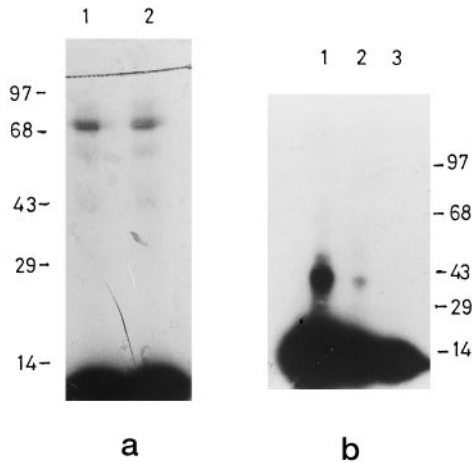

FIG. 7. **UV cross-linking analysis of the DNA- protein complex obtained with 0.5 M NaCl eluate of the heparin-agarose column and labeled positive element.** The experimental details are given in the text. *a*, the 0.5 M eluate was used to bind with labeled positive element and the mixture exposed to UV and then analyzed on SDS-PAGE (10% gel). *Lanes 1* and *2* are duplicates. *b*, complex III was specifically eluted after gel shift analysis and UV irradiation. The eluate was then analyzed on SDS-PAGE (10% gel). *Lanes 1* and *2*, complex III from two different experiments. Preparation *1* had five times more radioactivity than preparation *2; 3*, free DNA. The molecular weight markers are indicated.

results are obtained when the purified protein is used with nuclear extracts prepared from $CoCl_2$-treated rats (data not presented). Addition of BSA to crude extract does not elicit a similar response, indicating that the heme-dependent increase in binding to the positive element is specific to the 65-kDa protein (Fig. 9*c*). It is interesting to note that complexes II and III but not complex I are manifest with 0.5 M NaCl-heparin eluate of the $CoCl_2$-treated extract. Once again, complex II shows maximum response with hemin addition, although complex III also manifests some response. In addition, with the heparin column eluate maximum response is seen with 10^{-9} M hemin, whereas the optimal hemin concentration for crude extract as already indicated is 10^{-7} M. The purified ~65-kDa protein also shows a maximum response around 10^{-8} to 10^{-9} M hemin concentration for binding to the positive cis-acting element. This depends on the amount and source of crude nuclear extract added, uninduced or $CoCl_2$-treated, along with the 65-kDa protein to elicit the response. It is found that higher concentrations of hemin (10^{-6} to 10^{-5} M) are inhibitory. Thus,
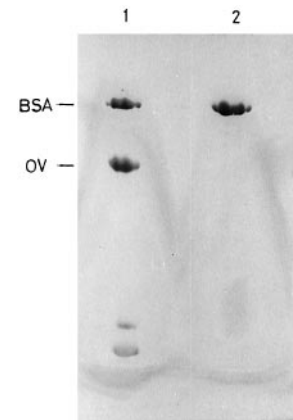
a higher concentration of hemin (10^{-7} M) is tolerated at the level of crude extract, since there are other proteins that may be able to compete for binding. As the protein is purified the competing proteins are removed and therefore the optimum hemin concentration to facilitate maximum binding gets lower.

It was then of interest to examine whether the heme-affinity column purified protein actually binds heme. For this purpose, the preparation was incubated with [^{59}Fe]-labeled hemin and then analyzed on SDS-PAGE, omitting the boiling step and addition of β-mercaptoethanol. A radioactive complex with a mobility of ~65 kDa is clearly seen on the autoradiogram (Fig. 10) indicating that the protein binds heme. The protein-[^{59}Fe]-labeled hemin complex was also fractionated on a Sepharose 4B column, which indicates that the heme-binding proteins migrates in the region of a BSA standard of mass of 65 kDa (Fig. 10*b*).

Finally, the purified preparation was examined for its potential to manifest heme-responsive stimulation of the transcription of pP450e179 DNA in cell-free extracts. For this purpose, the crude transcription extract was prepared from nuclei of $CoCl_2$-treated rats. The results presented in Fig. 11 (*a* and *b*) on the basis of laser densitometric analysis reveal that addition of hemin (10^{-8} M) or the purified protein (2 μg) to the transcription extract shows about 3- and 4–5-fold stimulation of transcription, respectively. The addition of the two together shows nearly 12-fold stimulation (Fig. 11*a*). There is also a significant graded response to the addition of increasing concentrations of the purified protein in the presence of hemin (Fig. 11*b*).

DISCUSSION

The present study clearly reveals that $CoCl_2$ treatment leads to a striking inhibition of CYP2B/B2 gene transcription that is overcome by the administration of low concentrations of hemin. The basal (uninduced) as well as PB-induced CYP2B1/B2 mRNA levels are also depressed by $CoCl_2$ treatment and counteracted by hemin administration (Fig. 1). Run-on and cell-free transcription studies indicate that $CoCl_2$ inhibits the PB-mediated increase in transcription and this is counteracted by the administration of exogenous hemin (Figs. 2 and 3). Cell-free transcription studies have indicated that the heme response element is within the 179 nt of the immediate 5′-upstream region of the CYP2B1/B2 gene.

Gel shift analysis with the positive element (−69 to −98 nt) correlates well with the inhibitory and counteracting effects of $CoCl_2$ and hemin, respectively, on CYP2B1/B2 mRNA levels and transcription status. As already reported (4, 12) crude

FIG. 9. **Binding of 65-kDa protein with labeled positive element.** The conditions for gel shift assay are described in text. *a*, effect of 65-kDa protein on binding. *Lanes 1*, crude extract (100 ng of protein); *2*, purified protein (~2–3 ng); *3*, crude extract + purified protein; *4–6*, same as *3* plus 10^{-7}, 10^{-8}, or 10^{-9} M hemin added *in vitro*, respectively. *b*, repeat experiment in *a*. *Lanes 1*, crude extract (100 ng of protein); *2*, purified protein (2–3 ng); *3*, crude extract+ 10^{-9} M hemin; *4*, crude extract + purified protein; *5*, crude extract + purified protein + hemin 10^{-9} M. *c*, effect of BSA on binding. *Lanes 1*, crude extract (100 ng of protein); *2*, crude extract + BSA (1 μg); *3* BSA (1 μg); *4–6*, same as *3* plus 10^{-7}, 10^{-8}, or 10^{-9} M hemin, respectively.
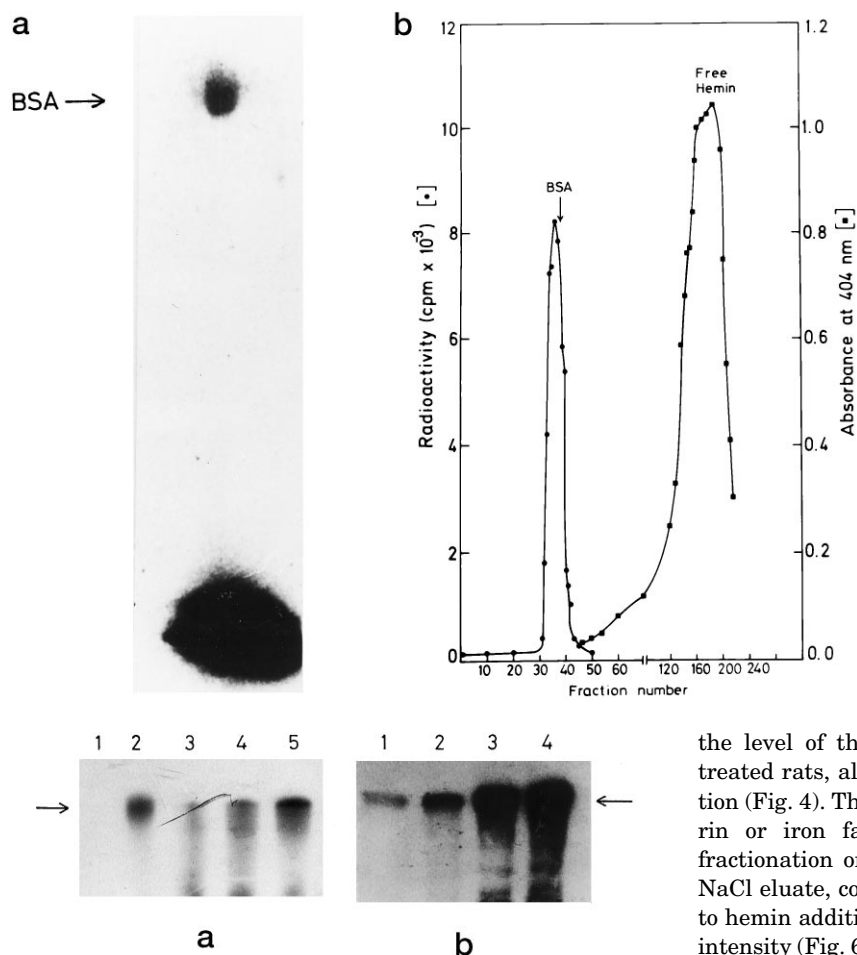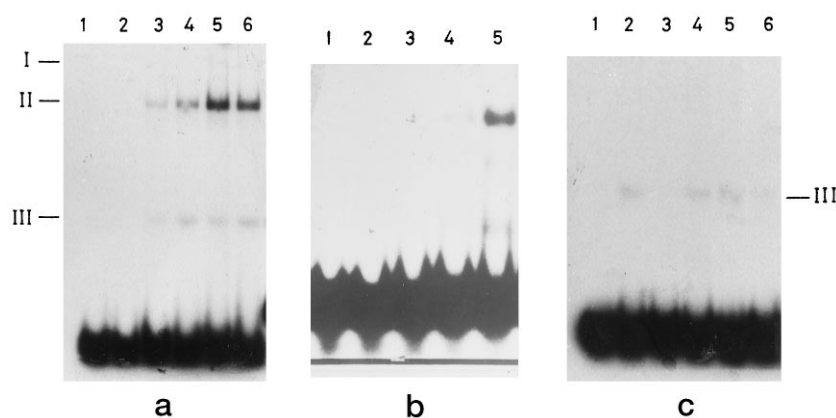

a b c

FIG. 10. **Binding of the 650-kDa protein to ^{59}Fe-labeled hemin.** The purified protein (~50–60 ng) was incubated with [^{59}Fe] labeled hemin (10 μCi/mg) as described in text. *a*, the complex was analyzed on SDS-PAGE (10% gel) omitting β-mercaptoethanol and the boiling step. *Arrow* indicates mobility of bovine serum albumin on the autoradiogram. *b*, the complex was fractionated on a Sepharose 4B column (22 × 1.7 cm) and 1-ml fractions were collected for measurement of radioactivity. The column was also separately calibrated with BSA and free [^{59}Fe] labeled hemin.

FIG. 11. **Effect of hemin and 65-kDa protein addition on the transcription of pP450e179 DNA in cell free extracts.** Transcription extracts were prepared from CoCl$_2$-treated rat liver nuclei as described in text. The transcripts were analyzed by RNase protection assay using exon I riboprobe. Treatments represent additions to the transcription mixture. *Arrow* indicates the position of the protected fragment of expected size in the autoradiogram. *Lanes 1*, complete reaction with extract (no addition of hemin or purified protein); *2*, probe alone. *3*, 10^{-8} M hemin; *4*, ~2 ng of 65-kDa protein; *5*, 10^{-8} M hemin + ~2 ng of 65-kDa protein. *b*, duplicate experiment. *Lanes 1*, 2 ng of 65-kDa protein; *2–4*, 10^{-8} M hemin + 2, 4, or 6 ng of 65-kDa protein, respectively. *Arrows* indicate the position of the protected fragment of expected size.

nuclear extract gives three complexes (I, II, and III) with the positive element. The present study reveals that CoCl$_2$ treatment leads to a suppression of formation of all the three complexes both in the presence and absence of PB treatment. At

the level of the crude nuclear extract isolated from CoCl$_2$-treated rats, all the three complexes intensify on hemin addition (Fig. 4). The effect is specific for hemin, since protoporphyrin or iron fail to elicit a similar effect (Fig. 5). After fractionation on the heparin-agarose column, with the 0.5 M NaCl eluate, complex II shows a striking increase in intensity to hemin addition and complex III shows a modest increase in intensity (Fig. 6). UV cross-linking analysis indicates that complex III consists of the ~26-kDa protein (Fig. 7). This is in agreement with the results reported from this laboratory (12), where the ~26-kDa protein purified from the 0.4 M NaCl eluate of the heparin-agarose column on the positive element oligonucleotide affinity column, has been shown to give rise to complex III with the positive element in gel shift analysis. The complex is faint, but in the presence of low amounts of crude nuclear extract or on concentrations of the purified fraction all the three complexes (I, II, and III) are manifest. The present study also reveals that complex II consists of the ~65-kDa protein, although traces of lower molecular weight proteins are also seen on UV cross-linking analysis. This is corroborated by the findings that the 0.5 M NaCl eluate of the CoCl$_2$-treated nuclear extracts from the heparin-agarose column gives a striking response to hemin addition in terms of generating complex II with the positive element (Fig. 6) and that a ~65-kDa heme-
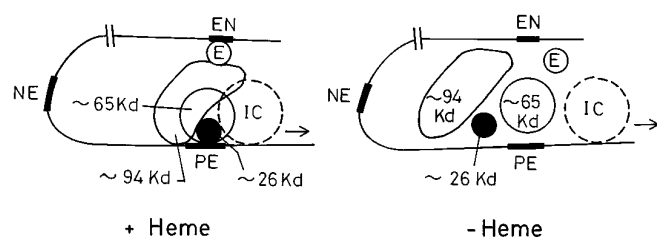
FIG. 12. **A model depicting the role of heme in the interaction of proteins with the positive element.** *PE*, positive element; *NE*, negative element; *E*, enhancer-binding protein; *EN*, enhancer; *IC*, initiation complex.

binding protein can be purified from this fraction using oligonucleotide and heme affinity columns (Figs. 8 and 10). As already indicated, the 0.5 M NaCl eluate from the heparin column also manifests heme-dependent generation of complex III, although complex II is dominant. It may be pointed that traces of low molecular mass proteins can be seen in the purified 65-kDa preparation. Once again binding of the purified ~65-kDa protein to the positive element is very poor and significant binding in the presence of hemin to the element is seen when small amounts of crude nuclear extract are added (Fig. 9). Thus, protein-protein interaction is essential for the binding of ~26- and ~65-kDa proteins to the positive element. UV cross-linking analysis of complexes II and III perhaps reveal only the proteins that make primary contact with positive elements and the other proteins involved in protein-protein interaction need to be identified.

In a recent study, it has been shown in this laboratory (12) that the ~26-kDa protein shows greater affinity to the positive (-69 to -98 nt) and negative (-126 to -166 nt) elements in its phosphorylated and dephosphorylated states, respectively, providing a basis for a differential interaction of the protein with the two elements in induced and uninduced states. While, the role of heme in the interaction of proteins to negative element is yet to be investigated, it is clear that interaction of the ~65-kDa protein with the positive element is heme dependent. Since, heme depletion leads to a decrease in basal as well as PB-induced transcription of the CYP2B1/B2 gene that is counteracted by the exogenous administration of hemin, it can be concluded that heme requirement is constitutive.

Earlier studies from this laboratory (3) have shown by Southwestern blot analysis that a ~94-kDa protein shows up when the entire 179 nt of the 5′-upstream region of the CYP2B1/B2 gene is used as a probe. PB treatment significantly enhances the intensity of this complex and this increase is blocked by $CoCl_2$ treatment of the animal. Thus, there are at least three proteins with molecular mass values of ~94, ~65, and ~26

kDa, whose interaction with the positive element and its neighborhood is influenced by the availability of heme and this in turn may influence the interaction of this protein assembly with the far upstream enhancer proposed by Ramsden *et al.* (11) as well as the initiation complex, leading to productive transcription of the CYP2B1/B2 gene. The ~65-kDa protein is perhaps the dominant protein that interacts with heme and facilitates interaction with other proteins and positive element. It needs to be studied whether heme has a direct role in the interaction of the ~26- and ~94-kDa proteins with cis-acting DNA elements. Fig. 12 depicts the model envisaged for the heme-dependent interaction of the proteins with the positive element. The ~26-kDa protein has already been shown to stimulate transcription of the minigene construct containing the positive element (pP450e179) in cell-free extracts (12). In the present study, the ~65-kDa protein has been shown to stimulate transcription of the pP450e179 DNA in cell-free extracts prepared from $CoCl_2$-treated rat liver nuclei in a heme dependent fashion. It, therefore, appears likely that the two proteins are positive regulators of the gene with binding sites on the positive element, although the possible interaction between the two needs to be studied.

REFERENCES

1. Hankinson, O. (1995) *Annu. Rev. Pharmacol. Toxicol.* **35,** 307–340
2. Matsushita, N., Sogawa, K., Ema, M., Yoshida, A., and Fujii-Kuriyama, Y. (1993) *J. Biol. Chem.* **268,** 21002–21006
3. Rangarajan, P. N., and Padmanaban, G. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 3963–3967
4. Upadhya, P., Rao, M. V., Venkateswar, V., Rangarajan, P. N., and Padmanaban, G. (1992) *Nucleic Acids Res.* **20,** 557–562
5. Ram, N., Rao, M. V., Prabhu, L., Nirodi, C. S., Sultana, S., Vatsala, P. G., and Padmanaban, G. (1995) *Arch. Biochem Biophys.* **317,** 39–45
6. He, J.-S., and Fulco, A. J. (1991) *J. Biol. Chem.* **266,** 7864–7869
7. Liang, Q., He, J-S., and Fulco, A. J. (1995) *J. Biol. Chem.* **270,** 4438–4450
8. Fournier, T., Mejdoubi, N., Lapoumeroulie, C., Hamelin, J., Elion, J., Durand, G., and Porquet, D. (1994) *J. Biol. Chem.* **269,** 27175–27178
9. Shephard, E. A., Forrest, L. A., Sherington, A., Fernandez, L. M., Ciaramella, G., and Phillips, I. R. (1994) *DNA Cell Biol.* **13,** 793–804
10. Trottier, E., Belzil, A., Stoltz, C., and Anderson, A. (1995) *Gene (Amst.)* **158,** 263–268
11. Ramsden, R., Sommer, K. M., and Omiecinski, C. J. (1993) *J. Biol. Chem.* **268,** 21722–21726
12. Prabhu, L., Upadhya, P., Ram, N., Nirodi, C. S., Vathsala, P. G., Mani, S. A., Rangarajan, P. N., Surolia, A., and Padmanaban, G. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 9629–9632
13. Ravishankar H., and Padmanaban, G. (1983) *Arch. Biochem. Biophys.* **225,** 16–24
14. Ravishankar, H., and Padmanaban, G. (1985) *J. Biol. Chem.* **260,** 1588–1592
15. Dwarki, V. J., Francis, V. N. K., Bhat, G. J., and Padmanaban, G. (1987) *J. Biol. Chem.* **262,** 16958–16962
16. Venkateswar, V., and Padmanaban, G. (1991) *Arch. Biochem. Biophys.* **290,** 167–172
17. Taylor, C. W., Yeoman, L. C., Dashal, I., and Busch, H. (1973) *Exp. Cell Res.* **82,** 215–226
18. Palmiter, R. D. (1973) *J. Biol. Chem.* **248,** 2095–2106
19. Guertin, M., Baril, P., Bartkowiak, J., Anderson, A., and Belanger, L. (1983) *Biochemistry* **22,** 4296–4302