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The Phenobarbital-Induced Transcriptional Activation of Cytochrome P-450 Genes Is Blocked by the Glucocorticoid-Progesterone Antagonist RU486

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SUMMARY

Several of the hepatic microsomal cytochromes P450 can be induced by various drugs and xenobiotics, among them the barbiturate phenobarbital. Rat hepatoma cells (Fao and its derivatives) respond to phenobarbital or dexamethasone treatment with an increased accumulation of CYP2C6 mRNA and thus provide a culture system to investigate the mechanisms involved. Examination of the kinetics of CYP2C6 mRNA induction revealed that the response to dexamethasone is rapid, whereas induction by phenobarbital occurs only slowly after an 8–10-hr lag. Run-on transcription measurements demonstrated that phenobarbital treatment led to a 3–4-fold increase in CYP2C6 gene transcription. Surprisingly, induction by phenobarbital of both accumulation of CYP2C6 mRNA and transcription of the gene was blocked by the antiprogestin-antiglucocorticoid RU486, suggesting the involvement of a steroid receptor in the induction process. Transfection of promoter constructs containing a reporter gene whose expression is driven by a 1.4-kilobase 5′ flanking segment of the CYP2B1 or CYP2B2 genes, which are highly inducible by phenobarbital in rat liver, led to >3-fold increases in reporter gene activity in the presence of the drug. Again, phenobarbital induction was prevented by RU486. The RU486 inhibition of the phenobarbital induction of both the endogenous CPY2C6 gene and the transfected CYP2B1 and CYP2B2 promoter constructs leads us to propose a model whereby the drug acts indirectly to cause the accumulation of an endogenous steroid, and this molecule, acting via its receptor, would be the direct inducer of cytochromes P450. Whether or not this model proves to be correct, the results presented here provide the first evidence of the involvement of a steroid receptor in phenobarbital induction.

The hepatic microsomal P450s are members of a superfamily of hemoprotein monooxygenases that function in the metabolism of a wide variety of endogenous substrates such as steroids, fatty acids, biogenic amines, and prostaglandins, as well as in the detoxification of many drugs and xenobiotics and in the activation of environmental agents to toxic, mutagenic, and carcinogenic forms (for reviews, see Refs. 1 and 2). There are >20 known distinct forms of rat hepatic microsomal P450s, which, in aggregate, are apparently capable of metabolizing thousands of substrates as a result of the distinct and broad, but partially overlapping, substrate specificities of specific enzymatic forms (2).

The individual P450s exhibit characteristic regulatory properties that include tissue specificity of expression, developmental and hormonal regulation, and inducibility by various drugs and xenobiotics (reviewed in Refs. 1 and 3). The inducibility is of great pharmacological significance, because the administration of an inducing drug can have a pronounced effect on the efficacy or toxicity of subsequent exposure to the same or other compounds. The inductive effect of the barbiturate PB has been studied for many years (reviewed in Ref. 4). This drug is now known to induce the expression of at least five distinct forms of rat hepatic P450 (1, 4). The PB induction phenomenon is of more general interest, because the drug also induces the expression of mRNAs encoding other enzymes that participate in drug detoxification, including aldehyde dehydrogenase, epoxide hydrolase, UDP-glucuronosyltransferase, and glutathione-*S*-transferase (1, 4). Interestingly, a wide variety of structurally dissimilar compounds have been shown to induce many of the same genes as PB (see, for example, Refs. 5–7), but a molecular explanation for this provocative phenomenon has not yet been provided.

A major impediment to the elucidation of the mechanisms responsible for the PB induction of P450 genes has been the

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ABBREVIATIONS: P450, cytochrome P450; PB, phenobarbital; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kb, kilobase(s); bp, base pairs(s); PCR, polymerase chain reaction; TAT, tyrosine aminotransferase; DEX, dexamethasone; CAT, chloramphenicol acetyltransferase.

failure to identify permanent liver-derived cell lines that manifest this induction. Such cells are necessary for transfection experiments using chimeric gene constructs to identify DNA segments (PB response elements) that mediate the induction of these genes. Recently, we reported (8, 9) that differentiated clonal hepatoma cell lines derived from the Reuber H35 hepatoma express several of the PB-inducible P450s. In particular, the Fao cell line and some of its derivatives displayed a 10-fold induction by PB of the mRNA encoding P45O2C6. This cytochrome is present at substantial levels in the livers of untreated rats and, in intact animals, undergoes a 2–4-fold induction after PB administration (9–11). In Fao cells, CYP2C6 mRNA levels were also increased 10-fold after administration of the synthetic glucocorticoid DEX, but simultaneous treatment with DEX and PB did not lead to higher levels of this mRNA than obtained with PB alone (9).

Surprisingly, Fao cells also contain substantial constitutive levels of the mRNA that encodes P4502B1 (8, 9), the major PB-inducible P450 of rat liver that is nearly undetectable in livers of untreated rats and is induced several hundredfold when rats are treated with PB (12, 13). In Fao cells, however, P4502B1 mRNA levels increase little, if at all, after PB treatment (8, 9).

In this paper we examine several additional aspects of PB induction of the CYP2C6 gene in Fao cells. We demonstrate that PB leads to transcriptional activation of the CYP2C6 gene and that this and the ensuing accumulation of CYP2C6 mRNA are blocked by simultaneous administration of the glucocorticoid-progesterone antagonist RU486. In addition, in cells transfected with recombinant plasmids in which a reporter gene is linked to the CYP2B1 or CYP2B2 promoters, PB treatment induces expression of the reporter gene and this induction is blocked by RU486. Based on these and other data, we propose a model whereby PB acts indirectly to induce expression of P450 genes by leading to intracellular accumulation of an endogenous steroidal compound that is the immediate inducer of the genes.

Materials and Methods

Cell culture. Fao rat hepatoma cells, originally derived from the H4IIEC3 Reuber hepatoma cell line (14), present the properties of well differentiated liver cells. FGC4 cells are derived from Fao and manifest the same differentiated phenotype (15) but can be transfected much more efficiently than Fao. Cells were cultured as described (16).

Inducers and anti-inducers. PB was obtained from Specia (Rhône Poulenc, Paris, France) and DEX from Sigma Chemical Co. (St. Louis, MO); RU486 was a gift from Roussel Uclaf (Romainville, France). DEX and RU486 were dissolved in ethanol and the final concentration of this vehicle was <0.1% (v/v), an amount that had no effect on the expression of the CYP2C6 gene. PB was used at a concentration of 2 mM because a dose-response curve revealed that half-maximal induction was obtained only in the millimolar range. Benzanthracene treatment was carried out as described (9).

Northern blot analysis. Total cellular RNA was prepared and analyzed as described previously (8), using CYP2C6 cDNA (plasmid pTF2) (11) as a hybridization probe. In some cases a larger (1.2-kb) CYP2C6 cDNA fragment (17), extending at its 3′ end to the natural *Eco*RI site in the coding sequence, was used as a probe. Isolated inserts of the recombinant plasmids were labeled by the random priming procedure (18) using [α-^{32}P]dCTP as substrate. A 20-nucleotide oligonucleotide probe (5′-AAATCCATTGAAAACTGGAG-3′) complementary to residues 1414–1395 of the published sequence of CYP2C6 mRNA (17) was also used as a specific probe for CYP2C6 mRNA. It was 5′-end labeled with polynucleotide kinase, using [γ-^{32}P]ATP (3000 Ci/mmol) as substrate, and was hybridized to the blots as described (19). When indicated, the hybridized DNA was quantified using a Phosphorimager and Imagequant software package (Molecular Dynamics, Sunnyvale, CA). In this case, the hybridization signals were normalized to those obtained with a GAPDH probe (20) in a second round of hybridization, after removal of the hybridized CYP2C6 probe by washing of the filter twice at 80° in 0.015 M NaCl, 0.0015 M Na Citrate.

PCR analysis of mRNA. This was carried out as described (21), using as primers a sense oligonucleotide corresponding to residues 1050–1069 (5′-CCAGAGGTTCATTGACCTCA-3′) of the reported CYP2C6 mRNA sequence (17) and the antisense oligonucleotide used as a hybridization probe. The amplified product was analyzed by electrophoresis on a 4% agarose gel, with and without prior digestion with *Eco*RI.

Run-on transcription analysis. This was carried out as described previously (22), using Hybond filters (Amersham) on which 10 μg of plasmid DNA containing cDNA inserts of either CYP2C6 (large *Eco*RI fragment) (17), CYP2B1 (23), TAT (24), β-actin (25), or GAPDH (20) or of pBluescript plasmid DNA had been immobilized. After hybridization, the filters were exposed to a Phosphorimager screen and the hybridization signals were quantified using Imagequant software. For each nuclear RNA sample, the background hybridization to the pBluescript DNA was subtracted from the amount of hybridization to each cDNA and the difference was normalized to the amount hybridized to GAPDH cDNA, which was arbitrarily set at 100. The value thus obtained was then expressed relative to the transcription rate for that gene in untreated cells, which was set at 100, to obtain the induction ratios resulting from the drug treatments.

Preparation of promoter constructs. The CYP2B2-luciferase plasmid (CYP2B2-Luc) was constructed from the plasmid pUC1.4-CAT (26, 27), which contains the 1.4-kb 5′ flanking segment of the CYP2B2 gene extending from the *Hind*III site at −1.4 kb to just upstream of the initiation codon. pUC1.4-CAT was linearized with *Sph*I, which cleaves at position −1, and was ligated to a synthetic double-stranded oligonucleotide containing the complete 5′ untranslated region of the gene preceded by a sticky *Sph*I site and followed by a *Hind*III site. After this ligation, the DNA was cleaved with *Hind*III, and the released 1.4-kb promoter fragment was isolated by agarose gel electrophoresis and cloned into the *Hind*III site of the promoterless luciferase plasmid pGL2-Basic (Promega, Madison, WI). A recombinant plasmid containing a 10-kb genomic *Eco*RI fragment that spans the transcription start site of the CYP2B1 gene and extends approximately 5 kb upstream was kindly provided by Dr. Johann Doehmer (Johannes Gutenberg University, Mainz, Germany). This was cleaved with *Sph*I, and the *Sph*I fragment extending from approximately position −4000 to nucleotide −1 was cloned into the *Sph*I site of pBLCAT3 (28), which was kindly provided by Dr. B. Luckow (German Cancer Research Center, Heidelberg, Germany). The resultant plasmid was cleaved with *Hind*III to remove genomic sequences upstream of −1.4 kb, and the plasmid was recircularized to form the CYP2B1(−1.4-kb)CAT construct. The CYP2B1-luciferase plasmid (CYP2B1-Luc) was derived from this plasmid by exactly the same procedure used to construct CYP2B2-Luc from pUC1.4-CAT, because the CYP2B1 and CYP2B2 genes have identical sequences in their 5′ untranslated regions and have *Hind*III and *Sph*I sites at identical positions in their 5′ flanking regions. The CYP2C6(−1226)CAT construct[2] contained, in the *Bgl*II site of pBLCAT3, a 5′ flanking segment of the CYP2C6 gene that encompassed residues −1226 to +13, derived from a genomic clone (29) kindly provided by Dr. Frank Gonzalez (National Institutes of Health, Bethesda, MD).

Transfection experiments. Cells (1×10^6) were plated in 10-cm culture dishes the day before transfection. The calcium phosphate precipitation procedure (30) was employed for transfection using 10 μg

[2] Shaw, P. M., M. C. Weiss, and M. Adesnik. Cooperative role of hepatocyte-enriched transcription factors, including HNF3, family members, in regulating the CYP2C6 gene. Manuscript in preparation.

of the promoter-reporter plasmid DNA and 1 mg of a cytomegalovirus-*lacZ* plasmid (pCMVβ, kindly provided by J. E. Darnell, Rockefeller University, New York, NY), which served as an internal standard, added to the cells for 16 hr. The inducers were added in fresh medium at the time of removal of the DNA precipitate. The cells were harvested 48 hr later and CAT and β-galactosidase activities were assayed as described (31). Luciferase activity was measured in lysates prepared in the same manner as those prepared for the CAT assay, using the Enhanced Luminescence assay kit and a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). The CAT or luciferase activity of each sample was divided by the β-galactosidase activity of the sample, to correct for differences in transfection efficiency, cell concentration, and protein recovery.

Results

PB-induced mRNA, hybridizes to a CYP2C6 gene-specific oligonucleotide probe, and PCR demonstrates that the correctly spliced form of the mRNA is expressed. We have previously (9) used a partial CYP2C6 cDNA (11) as a hybridization probe to demonstrate the PB-induced accumulation of CYP2C6 mRNA in Fao cells. The CYP2C P450 gene subfamily contains at least seven distinct members, which display approximately 50–75% sequence similarity to each other and are expressed in rat liver (32). We have observed that a cDNA for P4502C7 (plasmid pTF1) (11), which displays a higher sequence similarity (75%) (17) to CYP2C6 cDNA than does any other known member of the P4502C subfamily, does not hybridize to RNA from Fao cells under conditions in which strong hybridization with CYP2C6 cDNA is observed (data not shown). Although this observation indicates that hybridization of the CYP2C6 probe to Fao cell RNA almost certainly detects only CYP2C6 mRNA, we confirmed this conclusion by employing two additional analytical methods. Firstly, instead of a cDNA probe, we used as a hybridization probe a 20-nucleotide synthetic oligonucleotide that has at least four, and in some cases as many as 10, mismatches with the corresponding region of all other known members of the CYP2C subfamily of the rat. As expected (Fig. 1), PB treatment of Fao cells led to a marked accumulation of mRNA that hybridized to this CYP2C6-specific oligonucleotide probe.

Two alternatively spliced mRNAs derived from the CYP2C6 gene have been detected in RNA prepared from rat liver (33, 34). One of these encodes the normal functional P450, whereas the second is missing the normal eighth exon and contains instead a normally intronic sequence of similar length; it encodes a protein that is missing amino acid residues known to be critical for enzymatic activity (34). In a second method to characterize the CYP2C6-related mRNA expressed in Fao cells, we carried out PCR amplification of cDNA generated from Fao RNA, using a pair of primers corresponding to sequences in exons 7 and 9, and analyzed the PCR product for the presence of an *Eco*RI site contained in the exon 8 region of the normal mRNA but absent from the alternatively spliced mRNA. Fig. 2 shows that, whereas RNA preparations from the livers of either control or PB-treated rats yielded a substantial amount of amplified product that was not cleaved by *Eco*RI, mRNA from untreated Fao cells or from cells induced with PB or DEX was entirely cleaved by this restriction endonuclease. The sizes of the amplified product and of the fragments generated by restriction endonuclease digestion provide strong evidence that normal CYP2C6 mRNA molecules comprise the bulk of the transcripts in untreated and drug-treated Fao cells.

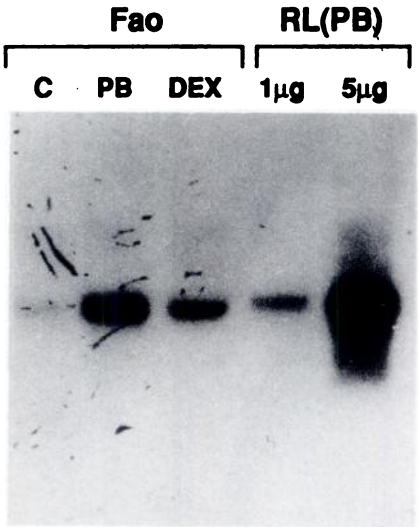

Fig. 1. PB induction of CYP2C6 mRNA and hybridization to a gene-specific oligonucleotide probe. Fao cells were treated with DEX (1 μM) or PB (2 mM) for 24 hr or left untreated to serve as a control (*C*). RNA (10 μg) extracted from the cultures was analyzed by Northern blotting, in parallel with 1 and 5 μg of RNA from the livers of rats treated with PB (100 mg/kg) for 16 hr [*RL(PB)*], using as probe a gene-specific oligonucleotide described in Materials and Methods.

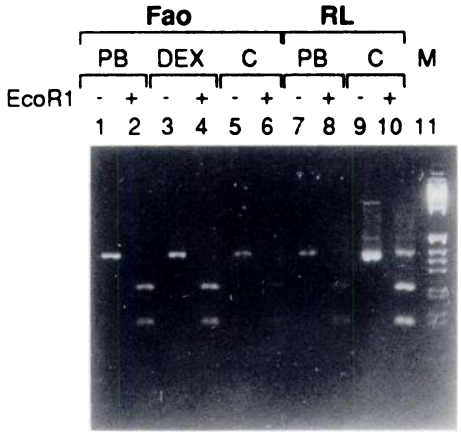

Fig. 2. The correctly spliced form of CYP2C6 mRNA is expressed in Fao cells. The same RNA samples analyzed in Fig. 1 and a sample of RNA from the livers of untreated rats (*RL*, *C*) were subjected to reverse transcription followed by PCR, and the amplified products were analyzed by gel electrophoresis with (+) and without (−) prior digestion with *Eco*RI. Note that only with rat liver RNA as template for reverse transcription does one detect, after *Eco*RI digestion of the amplified DNA, a band that migrates slightly more slowly than the bulk of the amplified product and is not cleaved by the enzyme. Also note that the reverse transcription PCR procedure, as used here, is not quantitative, so that the bands obtained with liver RNA from control rats are more intense than those obtained with the PB sample. *M*, molecular weight markers.

There is a marked lag in the accumulation of CYP2C6 mRNA after PB treatment. We next examined the kinetics of accumulation of CYP2C6 mRNA in Fao cells induced with either PB or DEX. Glucocorticoid treatment led to a rapid accumulation of CYP2C6 mRNA, with substantial levels being present as early as 4 hr after hormone addition (Fig. 3A). In striking contrast, the induction by PB took place only after a long lag; 8–12 hr after the addition of PB, the increase in CYP2C6 mRNA levels was only a small fraction of the maximum increase observed at 16 hr (Fig. 3B). The lag in the PB induction suggests that this drug acts indirectly to induce the

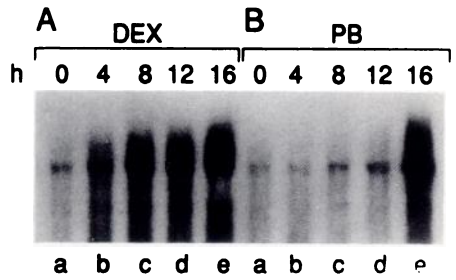

Fig. 3. Time course of CYP2C6 mRNA induction. Fao cells were treated with DEX (1 μM) (A) or PB (2 mM) (B) for the times indicated, and the extracted RNA (15 μg) was analyzed for CYP2C6 mRNA levels by the Northern blotting procedure, as described in Materials and Methods.

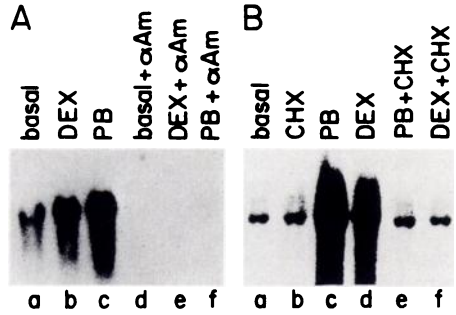

Fig. 4. Induction by DEX or PB is dependent on *de novo* RNA and protein synthesis. A, Fao cells were incubated with α-amanitin (*αAm*) (5 μg/ml) for 2 hr before addition of 1 μM DEX or 2 mM PB and an additional 16-hr incubation. Control cultures received no addition (*basal*) or α-amanitin, DEX, or PB alone, as indicated. RNA was extracted and analyzed as in Fig. 3. B, Fao cells were incubated with cycloheximide (*CHX*) (2 μg/ml) for 2 hr before addition of 1 μM DEX or 2 mM PB and an additional 16-hr incubation in the presence of these inducers. Control cultures received no addition (*basal*) or were treated with cycloheximide, DEX, or PB alone, as indicated. RNA was extracted and analyzed as in Fig. 2.

CYP2C6 gene, whereas the rapid DEX response is more compatible with a direct action of the hormone.

PB leads to transcriptional activation of the CYP2C6 gene. We next considered the question of whether the induction of CYP2C6 mRNA is a consequence of transcriptional activation. We first used the indirect approach of examining the effect of drugs that inhibit RNA synthesis on the induction of this mRNA. α-Amanitin, an inhibitor of transcription, completely blocked the induction by either inducer (Fig. 4A). In addition, the 16-hr treatment with this drug led to a marked diminution in the basal level of the CYP2C6 mRNA, indicating that the mRNA was unstable, so that its continued synthesis was required to maintain basal levels.

We next determined directly, using a run-on transcription assay with isolated nuclei, the effect of PB treatment on the rate of CYP2C6 gene transcription. In each experiment the extent of hybridization of *in vitro* labeled nuclear RNA to immobilized CYP2C6 cDNA (or to cDNAs corresponding to other specific mRNAs) was normalized to the amount that hybridized to the cDNA for the housekeeping gene GAPDH. It can be seen (Fig. 5; Table 1) that PB treatment led to a 2.7–4.4-fold increase in CYP2C6 transcription but had no effect on transcription of the β-actin or TAT genes. On the other hand, as expected, DEX did lead to marked transcriptional activation of the TAT gene while having no significant effect on actin gene transcription. It is noteworthy that the basal rate of CYP2B1 plus CYP2B2 transcription was substantially higher than that of CYP2C6 gene transcription (Fig. 5) and that this

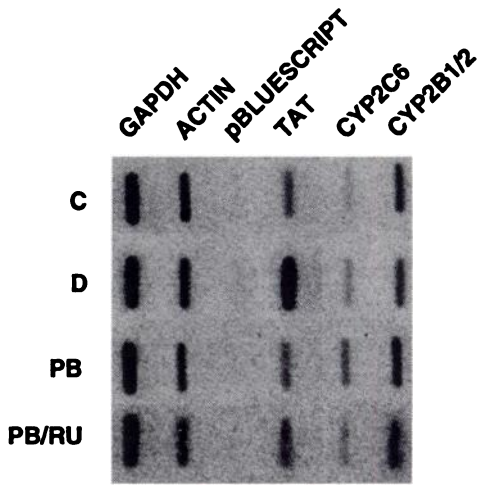

Fig. 5. PB treatment leads to transcriptional activation of the CYP2C6 gene that is blocked by RU486. Nuclei from untreated Fao cells (*C*) or cells treated with DEX (*D*) (1 μM), PB (2 mM), or PB in combination with RU486 (*RU*) (20 μM) for 14 hr were incubated *in vitro* with [^{32}P]UTP to produce labeled run-on transcripts, which were extracted and hybridized to filters containing the indicated slot-blotted cDNAs. pBluescript plasmid DNA served as a control. The filters were analyzed using a Phosphorimager. The data files from this exposure period were transferred to a TIFF file. Using the program Image 1.47b (available to the public on Internet via anonymous file transfer program from alw.nih.gov), the DEX, PB, and PB/RU486 filters were normalized to the GAPDH signal of the uninduced control filter. Quantitative data from these filters and from several other run-on transcription experiments, obtained by Phosphorimager analysis, are included in Table 1. In these experiments, the extent of PB induction of CYP2B1/2B2 transcription was marginal; at most a 30% increase was observed.

was not significantly increased by PB treatment. This mirrors the high basal level of the corresponding mRNA in Fao cells, compared with rat liver, and the failure of PB to substantially enhance this level (9). Although the extent of PB-induced transcriptional activation of the CYP2C6 gene was somewhat less than that expected from previous measurements of the effects of this inducer on mRNA levels, it should be noted that, in an experiment carried out in parallel with the run-on transcription measurements, PB led to only a 4.5–5.5-fold increase in CYP2C6 mRNA levels (Table 2). It appears from subsequent experiments that the PB induction ratio for these cells has now stabilized at approximately 5–6-fold.

The slow kinetics of induction of CYP2C6 mRNA accumulation after PB treatment would be consistent with a model in which PB acts indirectly by leading to the synthesis of a molecule, possibly a protein, that is required for transcriptional activation. To investigate this possibility, the effect of protein synthesis inhibition on the ability of PB and DEX to induce CYP2C6 mRNA was examined. It was observed that the addition of cycloheximide to cultures of Fao cells 2 hr before the addition of PB or DEX completely blocked the induction by either inducer, although it had no effect on basal mRNA levels (Fig. 4B). This indicates either that the induction by both compounds is an indirect process that requires the prior synthesis of an inducing protein or that a cooperating labile transcription factor is essential for expression or induction of the CYP2C6 gene (35). Because maintenance of the basal mRNA level requires continued RNA synthesis but not continued protein synthesis, it appears that protein synthesis is necessary only for inducer-mediated transcriptional activation of the gene and not for maintenance of its basal rate of transcription.

TABLE 1

RU486 blocks the PB-induced transcriptional activation of the CYP2C6 gene

Run-on transcriptional analysis was carried out with nuclei of cells treated with the various drugs for 4 or 14 hr, as indicated. The hybridized slot blots were analyzed using a Phosporimager, and induction ratios were calculated as described in Materials and Methods. In experiment 1, the GAPDH signals varied little, from approximately 20,000 to 27,000 pixels, whereas the CYP2C6 signals varied from approximately 700 (control) to approximately 1700 pixels (14 hr of treatment with PB). The nonspecific background hybridization to vector DNA was between 100 and 200 pixels. In the second experiment, the GAPDH signals were in the range of 2600–6200 pixels, the CYP2C6 signals between 70 and 140 pixels, and the nonspecific background hybridization to vector DNA in the range of 20–45 pixels, except for the 14-hr PB plus RU486 sample, in which the background of 65 pixels was slightly greater than the CYP2C6 signal (55 pixels).

Gene	Expt.	Induction Ratio								
		4 hr					14 hr			
		No treatment	DEX	PB	RU486	RU486/PB	DEX	PB	RU486	RU486/PB
		%								
CYP2C6	1	100	232	179		59	146	272		149
	2	100	478	207	62	122		439	200	0
TAT	1	100	465	106		109	812	90		112
	2	100	441	116	125	172		105		158
Actin	1	100	102	91		109	116	78		73
	2	100	71	128	88	87		75		63
GAPDH		100	100	100	100	100	100	100	100	100

TABLE 2

RU486 blocks the PB-induced accumulation of CYP2C6 mRNA

Cultures processed in parallel with those used for run-on transcription analysis were treated as indicated and analyzed by the Northern blotting procedure and Phosphorimager quantitation. The amount of hybridization to the radiolabeled CYP2C6 probe was normalized to that obtained with the GAPDH probe.

Treatment	CYP2C6 mRNA/GAPDH mRNA
Control	100
PB (4 hr)	131
DEX (14 hr)	550
PB (14 hr)	550
RU486 (14 hr)	82
DEX + RU486 (14 hr)	102
PB + RU486 (14 hr)	183
PB (24 hr)	449
PB (48 hr)	483

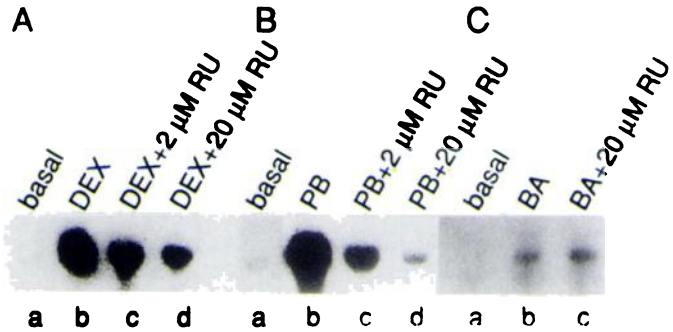

Fig. 6. RU486 blocks the induction of CYP2C6 mRNA by either DEX or PB. Fao cells were treated for 16 hr with either 1 μM DEX (A), 2 mM PB (B), or 1 mM benzanthracene (*BA*) (C), in conjunction with the indicated doses of RU486, before RNA extraction and analysis by the Northern blotting procedure, as in Fig. 1, using the CYP2C6 probe.

PB induction of CYP2C6 mRNA is blocked by RU486. The dose-response curve for the induction of CYP2C6 mRNA in Fao cells by DEX showed an ED_{50} of approximately 5×10^{-8} M,[3] a concentration that is at least 10-fold higher than that reported for TAT induction in the same cells (36), raising the possibility that the induction of CYP2C6 mRNA by DEX could occur by a mechanism that is different from the glucocorticoid receptor-dependent mechanism that mediates TAT induction. To determine whether the DEX induction of CYP2C6 mRNA is mediated by the glucocorticoid receptor, we examined the effect of the antiprogestin-antiglucocorticoid RU486 (37), a compound that prevents activation of the glucocorticoid receptor. It was observed that this antagonist did inhibit the DEX induction of CYP2C6 mRNA (Fig. 6A). As a control for the specificity of the antagonist inhibition of DEX induction, we also added RU486 to PB-treated cultures. To our surprise, this glucocorticoid-progesterone antagonist even more markedly inhibited the induction of CYP2C6 mRNA by PB (Fig. 6B). The inhibitory effect of RU486 on the PB induction of CYP2C6 was specific, because this steroid antagonist had no effect on the benzanthracene induction of P450IA1 mRNA in these cells (Fig. 6C). The unexpected inhibition by RU486 of PB induction suggests that induction by PB as well as DEX requires the action of the glucocorticoid (or progesterone) receptor, or possibly some as yet unidentified receptor that is encoded by a member of the steroid hormone receptor gene superfamily (38). Although PB itself could interact with such a receptor, it would seem more likely that this inducer acts either by leading to the accumulation of an endogenous sterol inducer or by potentiating the inducing effect of a steroidal compound present in the culture medium. The latter possibility was eliminated by the finding that PB induction of CYP2C6 mRNA in Fao cells is equally effective in the presence or absence of serum (data not shown). Moreover, PB does not induce either TAT mRNA (data not shown) or TAT gene transcription (Table 1).

To determine whether RU486 blocks the PB-induced accumulation of CYP2C6 mRNA at the transcriptional level, we carried out run-on transcription assays using nuclei from cells treated with various combinations of drugs. Treatment with RU486 caused a marked reduction in transcriptional activation by PB, when measured either 4 hr or 14 hr after administration of the drugs (Fig. 5; Table 1).

PB induces reporter gene expression in FGC4 cells transfected with CYP2B1 and CYP2B2 promoter constructs and this induction is blocked by RU486 treatment. Genomic clones containing the 5′ portion of the CYP2C6 gene and segments of the 5′ flanking region have been

[3] L. Corcos and M. C. Weiss, unpublished observations.

TABLE 3

RU486 inhibits the PB induction of luciferase expression in cells transfected with CYP2B1 and CYP2B2-luciferase constructs

FGC4 cells were transfected with CYP2B1-Luc, CYP2B2-Luc, or the promoterless pGL2-Basic plasmids, together with pCMVβ as indicated. The luciferase activity in each lysate was divided by the β-galactosidase activity to give the values shown. The induction ratios included in parentheses were obtained by normalizing these values, obtained from duplicate transfections, to the average value obtained for the duplicate control cultures. PB treatment had little effect (17–23% reduction) on the β-galactosidase activities of the transfected cultures; consequently, the PB-induced increases in the absolute values of the luciferase activity were very similar to the increases in the normalized values.

Treatment	Relative Luciferase Activity		
	CYP2B1-Luc	CYP2B2-Luc	pGL2-Basic
None	11,900; 11,200 (1.0)	2,670; 2,910 (1.0)	305; 287 (1.0)
Phenobarbital	37,000; 37,800 (3.24)	10,500; 9,680 (3.61)	252; 261 (0.85)
RU486	10,100; 10,100 (0.87)	2,930; 2,530 (0.97)	256; 200 (0.77)
PB + RU486	8,100; 8,560 (0.72)	2,890; 2,960 (1.04)	421; 302 (1.21)

isolated, and the sequence of 1226 bp of the 5′ flanking region was determined (29). Because the induction of CYP2C6 mRNA in Fao cells after PB or DEX treatment appeared to result from transcriptional activation, we carried out transfection experiments with CYP2C6 promoter-driven reporter gene constructs in which proximal promoter fragments of 1226 or 502 bp were inserted upstream of the bacterial (CAT) gene in the plasmid vector pBLCAT3. We found that the CYP2C6 promoter was highly active in Fao cells, and we obtained CAT activities that were even higher than those in cells transfected with albumin-CAT constructs. To our disappointment, however, the CAT activities in the cells with the CYP2C6-CAT plasmids were not reproducibly increased when the cells were treated with PB.

Previous studies showed that untreated Fao cells contain substantial amounts of CYP2B1 mRNA but no detectable CYP2B2 mRNA and that the CYP2B1 mRNA level is not significantly elevated after PB treatment (8). The transcription run-on data reported here (Fig. 5; Table 1) confirm this at the transcriptional level. Nevertheless, we decided to assess the activities of the CYP2B1 and CYP2B2 promoters in transfected hepatoma cells in the presence and absence of PB and, if a PB induction was observed, to determine to what extent it was affected by RU486 treatment.

We previously isolated a λ phage genomic clone containing the 5′ flanking region of the CYP2B2 gene (26, 27) and also obtained from Dr. J. Doehmer (Johannes Gutenberg University, Mainz, Germany) a λ clone containing the 5′ flanking region of the CYP2B1 gene. We used these clones to construct promoter-luciferase chimeric plasmids containing 1.4-kb proximal promoter fragments of the two P450 genes driving expression of the reporter gene, and we transfected these into FGC4 cells. In cells not treated with PB, both the CYP2B1 and CYP2B2 constructs showed substantial activity, approximately 38 and 9 times, respectively, that of the promoterless control plasmid (Table 3). Significantly, with both plasmids PB treatment led to a >3-fold increase in luciferase activity and these increases did not occur if the cells were simultaneously treated with RU486 (Table 3). PB treatment did not cause a measurable enhancement of luciferase activity in cells transfected with the promoterless plasmid pGL2-Basic. PB induction of reporter gene activity was also observed in cells transfected with a CYP2B1-CAT plasmid containing the 1.4-kb 5′ flanking promoter segment, and this too was eliminated by simultaneous exposure to RU486. In this case, in six separate transfections, a 3-fold induction was observed in the presence of PB (induction ratio, 2.9 ± 0.37), which was blocked by RU486 (induction ratio, 0.7 ± 0.19). One can conclude, therefore, that the steroid hormone antagonist effectively blocks the PB-induced transcriptional activation of the CYP2B1 and CYP2B2 promoters.

Discussion

The experiments reported here provide evidence that PB acts indirectly to induce expression of the CYP2C6, CYP2B1, and CYP2B2 genes. Firstly, accumulation of the CYP2C6 mRNA begins only 8–10 hr after Fao cells are exposed to the drug, in contrast to the rapid induction observed after DEX treatment. Secondly, cycloheximide fully prevents the PB induction of CYP2C6 mRNA, suggesting a requirement for protein synthesis. Cycloheximide treatment of rats was previously shown to block the transcriptional activation of the CYP2B1 and CYP2B2 genes by PB (39, 40), and we have recently observed the same inhibitory effect on the induction of the CYP2C6 gene in intact animals.[4] Thirdly, the PB induction of CYP2C6 transcription and the resultant accumulation of CYP2C6 mRNA, as well as the PB induction of reporter gene expression in cells transfected with CYP2B1 and CYP2B2 promoter constructs, are markedly inhibited when the antiprogestin-antiglucocorticoid RU486 is added simultaneously with PB. The inhibition is clearly specific, because the antagonist has no effect on the benzanthracene induction of P450IA1, which is mediated by the polycyclic aromatic hydrocarbon receptor (41).

These findings lead us to propose a two-step mechanism of PB induction (Fig. 7); PB treatment leads to the accumulation of an endogenous steroid-like compound, which then binds to its specific receptor to form a complex that directly activates expression of the CYP2C6 gene. The lag observed in PB induction could represent the interval during which the endogenous inducer accumulates. The RU486 inhibition of PB induction implicates the interaction of a steroid with its cognate receptor in the induction process, because the structural dissimilarities of PB and its antagonist RU486 make it unlikely that the latter directly competes with PB for binding to a PB receptor. We propose that this steroid is an endogenous cellular constituent, because PB induction occurs even in serum-free medium.[5]

A model whereby PB acts to induce P450 gene expression by leading to the accumulation of an endogenous steroid like-inducer is attractive for several reasons. Firstly, it has never been possible to detect a receptor that specifically binds PB, although this could be attributed to a low affinity of the ligand for its hypothetical receptor (6). Secondly, because PB is a

[4] A. Kumar and M. Adesnik, unpublished observations.
[5] M. C. Weiss, unpublished observations.

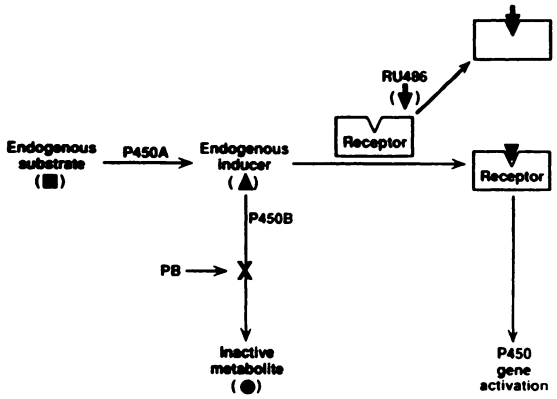

Fig. 7. Model for PB induction of P450s. An endogenous inducer (▲) is presumed to be normally inactivated by a specific P450 (P450B) that is inhibited by PB and other substrates and inhibitors of that P450. As a consequence of inhibition of that P450, the levels of the endogenous inducer increase and lead to induction via activation of a specific receptor. RU486 functions as an anti-inducer, because it can bind to that receptor but cannot activate it. It is presumed that the endogenous inducer, which is likely to be a steroid, is produced by a P450-dependent pathway, but this is not an essential feature of the model.

substrate for some P450 enzymes of family II that are also highly active in steroid hydroxylation (42), it is plausible that PB competitively inhibits an enzyme that metabolizes an endogenous steroidal substrate, thus leading to its intracellular accumulation. Our two-step model, however, is not compatible with the findings of He and Fulco (43) that incubation of rat liver nuclear extracts with PB resulted in the appearance of a new gel-shifted complex upon subsequent incubation with a 17-bp double-stranded oligonucleotide from the CYP2B1/2B2 proximal promoter region. That observation suggests a direct interaction between the inducer and a component of the nuclear extract. Our two-step model predicts that other substrates of the P450 enzyme that normally inactivates the endogenous inducer could cause a "PB-like" induction. This would explain the enigmatic observation that a wide variety of structurally dissimilar compounds induce many of the same genes as PB (see, for example, Refs. 3, 5, and 7) and hence are referred to as PB-like inducers.

Although the RU486 inhibition of PB induction implicates an endogenous steroid in the induction process, the steroid may play an indirect role and may simply be necessary for the induction to occur. On the other hand, the steroid could act directly, whereas the PB could act indirectly to sensitize cells to the endogenous inducer without affecting its cellular levels. For example, treatment of Fao cells with PB has been shown to sensitize cells to glucocorticoid by leading to a 30% increase in the cellular concentration of glucocorticoid receptors and to a 2-fold increase in the affinity of the receptor for DEX (44). However, the small magnitude of the effect of PB on the glucocorticoid receptor seems insufficient to account for PB induction of P450.

The concentration of RU486 required to antagonize the PB induction is quite high (approximately 20 μM), raising the possibility that the endogenous steroid that participates in the induction is present in the cells at a very high concentration or has an unusually high affinity for its receptor. Alternatively, RU486 could have a much lower affinity for the receptor with which the endogenous inducer interacts than it has for the glucocorticoid or progesterone receptors. The receptor to which the endogenous steroid binds could be a novel receptor, perhaps one of the so-called orphan receptors (with, as yet, unknown ligands) encoded by a member of the steroid hormone receptor gene superfamily (38).

The fact that a PB response is observed in the rat hepatoma cell lines (Fao and its derivative subclone FGC4) prompted us to analyze the promoters of the PB-inducible CYP2C6, CYP2B1, and CYP2B2 genes for their capacity to be activated by PB treatment. The CYP2C6 constructs, containing either a 505 bp or a 1.2-kb promoter fragment, showed high basal expression but not reproducible induction, indicating that a critical PB response element is absent from these fragments. In contrast, CYP2B1 and CYP2B2 constructs containing 1.4-kb promoter segments, which show low basal expression, led to a highly reproducible 3-fold induction after PB treatment.

At first these results appear paradoxical, because the promoter response does not mimic the behavior of the corresponding genes in the hepatoma cells. Rather, the behavior of the transfected genes is more similar to the response observed in liver, i.e., high constitutive expression of CYP2C6 and low basal expression coupled with PB inducibility of CYP2B1 and CYP2B2. However, the promoter response would be expected to be a faithful reflection of the transcription factor content of the cells (in fact, this is the underlying premise of *trans*-activation tests), whereas the endogenous genes are subject to other influences as well, such as methylation and chromatin structure. Moreover, in comparing the basal activity with the inducibility of these genes, a high basal expression appears to be correlated with low inducibility. Thus, examination of a panel of hepatoma clones related to Fao for P450 2C6 mRNA levels revealed that clones with lower basal levels showed higher induction ratios (9).[8] The same inverse correlation of CYP2C6 basal levels and the magnitude of the induction by DEX was observed in a panel of inbred mouse strains (45). In addition, CYP2C6 mRNA is induced by PB to a much greater extent in primary hepatocyte cultures than it is in the livers of adult rats (5). This appears to result from the fact that, after several days in culture, hepatocyte CYP2C6 mRNA levels decline to almost undetectable values but subsequent PB treatment leads to a marked increase to the *in vivo* basal levels. The high basal level of CYP2C6 expression in the liver, which is not increased after DEX treatment (45), could be a consequence of partial induction by circulating endogenous glucocorticoids, in line with the observation that DEX induces CYP2C6 mRNA in the hepatoma cells. Indeed, although both the CYP2C6 and CYP2B1 genes behave differently in adult rat liver and in the *in vitro* cultured hepatoma cells, it is perhaps unreasonable to expect that their *in vivo* regulatory behavior would reflect solely the inherent transcriptional capacity of the hepatocytes. In particular, the intact animals are subject to multiple and changing hormonal influences that could affect basal activities to enhance or depress them, as documented in numerous publications (46), and consequently also affect the fold induction.

The demonstration in the experiments presented here of the PB induction of luciferase expression in cells transfected with the CYP2B1- and CYP2B2-luciferase plasmids that contain 1.4-kb proximal promoter fragments represents an important breakthrough in our attempts to elucidate the molecular mechanism of the induction process, because it now becomes possible

[8] L. Corcos and M. C. Weiss, unpublished observations.

to map the DNA sequences that mediate the induction and to identify the factors that bind to these sequences. In this regard, it is worth noting that studies on the chicken PB-inducible CYP2H1 gene, employing chicken primary hepatocytes as hosts for transfection, have revealed the presence of a PB-responsive element between positions −5.9 and −1.1 kb, because a segment covering that region conferred PB responsiveness (14-fold induction) on an enhancerless SV40 promoter (47). Taken together, these analyses of the rat and chicken CYP2 genes provide two independent systems for elucidating the molecular mechanisms of PB induction.

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