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MOLECULAR PHARMACOLOGY, 38:462-470

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Developmental Expression and *In Situ* Localization of the Phenobarbital-Inducible Rat Hepatic mRNAs for Cytochromes CYP2B1, CYP2B2, CYP2C6, and CYP3A1

CURTIS J. OMIECINSKI, CHRISTOPHER HASSETT, and PAOLA COSTA
Department of Environmental Health, University of Washington, Seattle, Washington 98195
Received May 30, 1990; Accepted July 17, 1990

SUMMARY

In this study we examined the differential hepatic expression of four phenobarbital (PB)-inducible rat cytochrome P450 genes, CYP2B1, CYP2B2, CYP2C6, and CYP3A1. The mRNAs encoding these cytochromes were analyzed in the liver with respect to PB responsiveness, developmental expression, and *in situ* localization. Utilization of the polymerase chain reaction enabled assessment of specific hepatic mRNA expression patterns during early development that were not detectable with standard Northern blot or slot-blot procedures. The polymerase chain reaction data demonstrated that fetal liver from day 15 of gestation was responsive to the inductive effects of transplacental PB administration. Both constitutive and PB-induced levels of each mRNA increased with increasing developmental age, reaching maximal levels approximately 3 weeks postpartum. An exception to this trend was observed for rats of gestational day 22, which exhibited transiently increased constitutive levels of CYP2B1, CYP2B2, and CYP3A1 mRNAs, such that PB-induced levels were not elevated over those observed in untreated animals. *In situ* hybridization data, obtained with livers form 6-week postpartum animals, revealed striking differences in regional distributions among the cytochrome P450 transcripts. Whereas patterns of PB-induced expression of CYP2C6 mRNAs were relatively homogeneous across the hepatic lobule, CYP3A1 mRNAs in PB-treated livers demonstrated marked centrilobular localization. These results were in contrast to data obtained previously for PB-inducible CYP2B1 and CYP2B2 mRNAs, which were distributed homogeneously across the hepatic lobule except for cells in the immediate vicinity of the periportal tract.

PB is a prototype inducer of a number of mammalian genes, including microsomal epoxide hydrolase (1), certain glutathione transferases (2), NADPH-P450-oxidoreductase (3), UDP-glucuronosyltransferase (4), and some members of the P450 monooxygenase gene superfamily[1] (6, 7). Available data indicate direct transcriptional activation as the primary mechanism responsible for PB induction (8, 9); however, the molecular events associated with PB induction are not well elucidated.

In the rat, members of at least three distinct P450 subfamilies are induced by PB. For example, in the CYP2 family CYP2B1, 2B2, and 2C6 are PB responsive (7–12), and in the CYP3 family CYP3A1 and 3A2 are similarly induced by PB (13). The PB-responsive P450 genes also are subject to highly tissue-specific and developmentally regulated programs (11, 12, 14, 15).

CYP2B1 and CYP2B2 are >97% identical in primary sequence and also share highly similar genomic organization (16). Despite these similarities, the respective genes are differentially induced by PB in the adult liver (10, 11, 17), but the mRNA transcripts share the same zonal distributions within the liver lobule (18). Constitutively, low levels of only CYP2B2 are detectable in the Sprague-Dawley liver, whereas only CYP2B1 is evident in lung or testis preparations (11, 19). RNA analyses from transplacental induction experiments indicated that fetal liver also is responsive to PB, but only during late gestational stages (14, 15).

Based on primary nucleic acid sequence comparisons, CYP2C6 is approximately 50% related to CYP2B1/2 and 36% related to CYP3A1/2 (12, 13, 16). CYP2C6, expressed constitutively in the rat liver, is PB inducible but is induced to a much lesser degree than either the CYP2B or CYP3A subfamily members (12). The CYP3A1 gene (43% similar to CYP2B1/2) is recognized for its inducibility by dexamethasone (7, 13). P4503A2 shares 90% cDNA sequence homology with CYP3A1 but is not dexamethasone inducible (13). However, both 3A1 and 3A2 are highly responsive to PB in the rat liver (13).

The present study was undertaken to examine the following questions. 1) Can a PCR technique be developed to discriminate among highly homologous PB-inducible P450 mRNAs? 2) Would a PCR-based assay offer enhanced insight into P450 expression patterns in tissues and early developing organisms

This work was supported by Grants GM-32281 and ES-04696 from the National Institutes of Health.

[1] The nomenclature employed for P450 designations is as suggested by Nebert *et al.* (5).

ABBREVIATIONS: PB, phenobarbital; P450, cytochrome P450; PCR, polymerase chain reaction; bp, base pair.

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where expression was previously undetectable? 3) Are all PB-inducible P450 mRNAs, despite varying degrees of evolutionary relatedness, similar with respect to their spatial patterns of expression across the hepatic lobule? To address these questions, we selected the P450s 2B1/2, 2C6, and 3A1 for study, because they share the property of PB inducibility in rat liver, yet vary considerably in comparative primary sequence relatedness.

The data obtained demonstrated that PCR approaches can be employed to discriminately amplify highly related P450 mRNAs. With the increased sensitivity achieved by this technique, RNA expression patterns were ascertained that were not identifiable previously. Results of *in situ* hybridization studies suggested that, despite the common feature of PB inducibility shared by four unique P450 genes in the liver lobule, regional distributions of hepatocytes expressing the respective mRNA products are quite distinct.

Materials and Methods

Tissue sources and RNA isolation. Sprague-Dawley rats were obtained from Tyler Laboratories, Inc. (Bellevue, WA). To assess PB induction, pregnant rats were injected intraperitoneally with either 50 mg/kg PB or saline alone (controls) for 3 consecutive days, with the last dose given 16 hr before sacrifice; neonatal and adult rats were injected with 75 mg/kg PB 16 hr before sacrifice. For dexamethasone (Aldrich) treatments, an adult male and an adult female (46 days of age) were injected intraperitoneally with 100 mg/kg, 16 hr before sacrifice. Pregnant rats, beginning on day 18 of gestation, were injected twice, 24 hr apart, either with dexamethasone alone at 100 mg/kg or with a combination of 100 mg/kg dexamethasone and 50 mg/kg PB; the last dose was administered 16 hr before removal of the fetuses via cesaerean section. Rats were housed in plastic cages, provided access to food and water *ad libitum*, and maintained on a 12-hr light/dark cycle. The day following time-mated pregnancy was designated day 0 of gestation. The gestational period of the rat is 22 days. For prenatal time points, liver tissue from at least 10 fetuses obtained from three pregnant rats/treatment group was pooled for RNA isolation. For neonatal and adult animals, livers were pooled from three animals/treatment group. For these studies, RNA was isolated and Northern blot studies were performed according to procedures described previously (15, 20).

PCR reactions. PCR reactions were performed in 50-μl final volumes, consisting of 25 mM Tris·HCl, pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 0.2 mM levels of each deoxynucleotide triphosphate, 0.1 mg/ml bovine serum albumin, 20 pmol each of forward and reverse primer, 2 μl of cDNA, and 2 units of Taq polymerase (New England Biolabs). Following the addition of three drops of mineral oil (Sigma), the reactions were heated to 93° for 4 min and immediately cycled 30 times in a programmable heating block (Coy Laboratories) through a sequence consisting of 1-min denaturation at 93°, 1.5-min primer annealing at 54°, and 1-min polymerization at 72°. In some experiments, the PCR products were precipitated with ethanol in the presence of 5 μg of yeast tRNA. In most cases, 10 μl of the PCR reaction product were directly separated through a 2% NuSieve/1% SeaKem LE agarose mixture (FMC) and visualized by ethidium bromide staining. Sizes of the PCR products were estimated from the migration of DNA size markers (Drigest III; Pharmacia) run concurrently. Transfer of gels to GeneScreen Plus membranes (NEN/DuPont) was achieved by capillary blotting. Southern blotted membranes were probed with ^{32}P-labeled hybridization oligomers, targeted to internal regions of the expected PCR products, and exposed to Kodak XAR film. PCR amplifications and Southern hybridizations were repeated at least twice to establish reproducibility of results.

cDNA synthesis and oligonucleotide probes. Five micrograms of total RNA and 20 pmol of oligo $d(T)_{20}$ were dissolved in 12 μl of diethylpyrocarbonate-treated water/0.1 mM EDTA, heated to 75° for 2 min, and placed at 42°. Eight microliters of a buffer mix were then added, providing final reaction concentrations of 25 mM KCl, 6 mM $MgCl_2$, 10 mM dithiothreitol, 0.25 mM levels of all four deoxynucleotide triphosphates, 50 mM Tris·HCl, pH 8.3, 25 units of placental ribonuclease inhibitor (Amersham), and 17 units of avian myeloblastosis virus (AMV) reverse transcriptase (Seikagaku). Samples were incubated for 1 hr at 42°. Similar PCR results were also obtained when the respective antisense oligomers were substituted for oligo $d(T)_{20}$ as primers in the cDNA reactions (data not shown).

Oligomers were synthesized by the Howard Hughes Medical Institutes at the University of Washington, with an Applied Biosystems 380A synthesizer. The nucleotide sequences of the oligomers employed for PCR, Northern blot, and *in situ* analyses are indicated in Table 1.

The PCR forward and reverse primers for CYP2B1 and CYP2B2 were synthesized in the mRNA sense and selected to enable specific amplification of each respective cDNA in the region corresponding to the junction of exons 6 and 7. Each of the forward primers (20-mers) contained a mismatched base (2B1 versus 2B2) at their extreme 3′-positions and were targeted to different positions within the exon 6 region to allow for differentiation of the individual PCR products with respect to size. The CYP2B1/2 antisense reverse primers (25-mers) were targeted to a common region corresponding to exon 7 of each gene but contained four base mismatches at their respective 3′-ends. Hybridization probes (18-mers) were utilized to allow independent confirmation of PCR product identity through differential hybridization to sequences internal to the expected PCR products. The hybridization probes (antisense) for CYP2B1/2 differed in 4 of 18 positions and have been shown previously to recognize each of the respective sequences with a high degree of specificity (10, 11).

The CYP3A1 primers and hybridization probe sequences were chosen to provide discrimination between CYP3A1 and CYP3A2 mRNAs. The 3A1 oligomer sequences chosen each contain at least three mismatches when compared with the corresponding positions of 3A2 (13). Specifically, in this respect, the 3A1 20-mer forward primer contains four mismatches, the 20-mer reverse primer contains three mismatches, and the 22-mer hybridization probe harbors three mismatches. In some instances, the PCR products were subjected directly to DNA sequencing, and the sequences agreed with those of the predicted products (data not shown). The 2C6HP, RAT18SHP, and RATALBHP antisense sequences utilized were 20-mers derived from published cDNA sequence data for rat CYP2C6 (12), rat serum albumin (21), and rat 18S ribosomal RNA (22), respectively.

***In situ* hybridization.** *In situ* hybridization experiments were performed with untreated and PB-pretreated Sprague-Dawley rats, 60 days of age (approximately 6 weeks postpartum). Liver tissue sections, oligomer annealing, and post-hybridization washes were exactly as described previously (18). However, CYP3A1, 2C6, and albumin oligomer hybridization probes were labeled with terminal deoxyribonucleosidyl transferase (Pharmacia/LKB) and equimolar concentrations of α-^{35}S-deoxy-ATP (specific activity, 500 Ci/mmol; DuPont) and α-^{35}S-dideoxy-ATP (specific activity, 1320 Ci/mmol; DuPont). Probe was added to the hybridization solution at 0.015 pmol/ml (approximately 7 $\times$ 10^5 dpm/ml). This method of labeling resulted in an approximate 2-fold increase in probe specific activity, compared with that employed previously (18). The extent of autoradiographic exposure was variable, depending on the oligomer probe employed and target RNA population and is described in the figure legends.

Results

In the design of our PCR strategy, we selected DNA primers whose sequences and relative sites within the respective P450 molecules would enable 1) discriminative amplification of specific P450 cDNAs and 2) clear identification of PCR products, based on predicted size and hybridization with oligomer probes targeted to predicted sequences within the PCR products.

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TABLE 1
Summary of DNA oligomer sequences

Oligomer	Description	Sequence
CYP2B1FP	PCR forward primer	5′-GCTCAAGTACCCCCATGTCG-3′
CYP2B1RP	PCR reverse primer	5′-ATCAGTGTATGGCATTTTACTGCGG-3′
CYP2B1HP	Hybridization probe	5′-GGTTGGTAGCCGGTGTGA-3′
CYP2B2FP	PCR forward primer	5′-CTTTGCTGGCACTGAGACCG-3′
CYP2B2RP	PCR reverse primer	5′-ATCAGTGTATGGCATTTTGGTACGA-3′
CYP2B2HP	Hybridization probe	5′-GGATGGTGGCCTGTGGA-3′
CYP3A1FP	PCR forward primer	5′-CCGCCTGGATTCTGTGCAGA-3′
CYP3A1RP	PCR reverse primer	5′-TGGGAGGTGCCTTATTGGGC-3
CYP3A1HP	Hybridization probe	5′-CGGATAGGGCTGTATGAGATTC-3′
CYP2C6HP	Hybridization probe	5′-CCTCTGGACACTTCAGCAGG-3′
RATALBHP	Hybridization probe	5′-GCCTTGGGCTTGTGTTTCAC-3′
RAT18SHP	Hybridization probe	5′-CACCTCTAGCGGCGCAATAC-3′

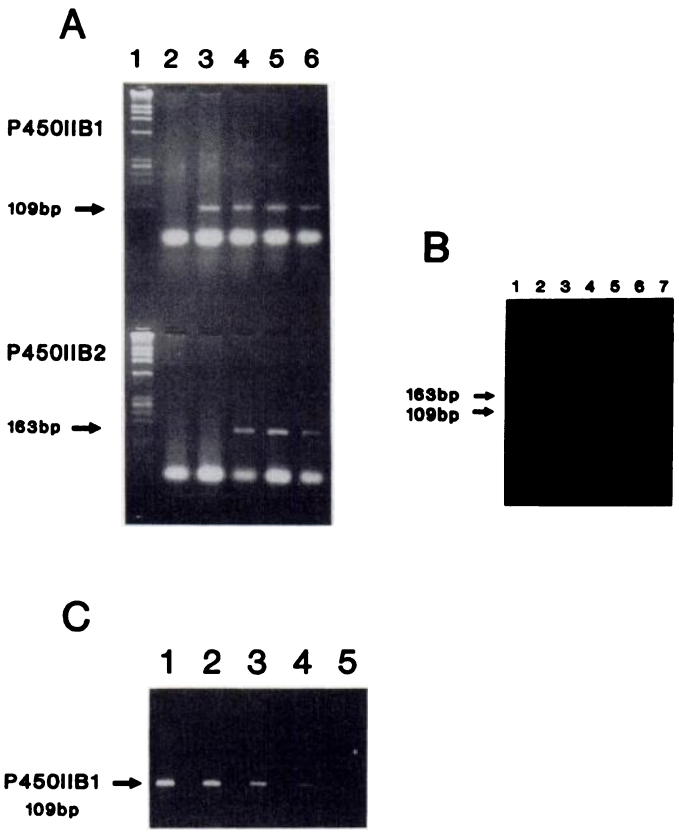

Fig. 1. Rat CYP2B1 and CYP2B2 PCR amplification products. RNA was isolated from various tissue sources and reverse transcribed and the resulting cDNA was subjected to amplification with specific primer sets. Ethidium bromide-stained agarose gels are shown. A, The upper gel contains PCR products of 2B1-primed reactions and the lower gel, products of 2B2-primed reactions. In each gel, *lane 1*, molecular size markers; *lane 2*, kidney; *lane 3*, lung; *lanes 4* and *5*, PB-induced liver; and *lane 6*, untreated liver samples. B, *Lane 1* molecular size markers; *lanes 2–5*, control liver samples amplified with 2B1 primer sets; *lanes 6* and *7*, control liver samples amplified with 2B2 primers. C, PCR products resulting from amplification of serially diluted cDNAs derived from PB-induced liver RNA. *Lane 1*, PCR products amplified from undiluted cDNA (the amount loaded was produced from the equivalent of 0.1 μg of total cellular RNA); *lane 2*, 1:10 dilution of cDNA sample employed in lane 1; *lane 3*, 1:100 dilution; *lane 4*, 1:1000 dilution; *lane 5*, $1:10^6$ dilution. *Arrows*, the 109-bp (2B1) and 163-bp (2B2) products.

In Fig. 1, results of PCR experiments are presented demonstrating discriminant amplification of the highly homologous CYP2B1 and CYP2B2 mRNA transcripts. Relative to DNA size markers in Fig. 1A, *lane 1*, the upper *gel* shows clearly the presence of a 109-bp band and the lower *gel*, a 163-bp band. The size of each band was consistent with that predicted for the CYP2B1 and CYP2B2 PCR products, respectively. In this particular experiment, tRNA was added as carrier before ethanol precipitation of the PCR products. The tRNA component is clearly visible as the low molecular weight bands in Fig. 1A. Results of another PCR experiment are presented in Fig. 1B, in which samples generated from either CYP2B1 or 2B2 amplification were loaded directly on the same gel for size analysis. Again, bands of 163 bp (Fig. 1B, *lanes 2–5*) and 109 bp (Fig. 1B, *lanes 6* and *7*) are apparent. To further establish the authenticity of the products, these gels were Southern blotted and probed with the respective 2B1 or 2B2 hybridization oligomers. These data (not shown) and the data from Fig. 2 demonstrated each of the identified CYP2B1 or CYP2B2 products to hybridize specifically to probes targeted to internal regions of the amplified sequence.

To examine whether PCR-derived data would corroborate results obtained previously for CYP2B1 and CYP2B2 in Northern blotting and solution hybridization studies (11, 15), PCR experiments were conducted with RNA isolated from different rat tissues and at various stages of rat liver development. In Fig. 1A the corresponding lanes of the upper and lower gels contain PCR products derived from the same cDNA sample; however, the upper gel was loaded with samples amplified in the presence of the CYP2B1 primers, whereas the lower gel contained reaction products obtained with CYP2B2 primers. Amplified kidney cDNA preparations were loaded in Fig. 1A, *lane 2*, which showed no detectable 2B1 or 2B2 products. These results were consistent with earlier data obtained with Northern blotting of rat kidney mRNAs (11). Lung cDNAs also were amplified and the resulting products were assessed in Fig. 1A, *lane 3*. The upper gel containing the 2B1 products exhibits a 109-bp band, but in the corresponding lane of the lower 2B2 gel only traces of 163-bp product are potentially present. However, upon Southern blot hybridization to the respective 2B1 and 2B2 probes (data not presented), only the 2B1 probe hybridized, indicating that little or no 2B2 mRNA is present in the Sprague-Dawley lung. The PCR results obtained with the lung preparations also are consistent with previous Northern blot data (11).

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Fig. 1A, *lanes 4–6* contain PCR products from three different liver preparations. Fig. 1A, *lanes 4* and *5* contain two different PB-treated liver samples and Fig. 1A, *lane 6* contains a sample from untreated liver. Strong bands of both 109 bp (upper gel) and 163 bp (lower gel) are evident in all the liver samples, indicating hepatic expression of both 2B1 and 2B2 mRNAs. In Fig. 1B, four additional untreated liver RNA samples were evaluated. The samples in Fig. 1B, *lanes 2–5* were amplified with the 2B1 primer pairs and the samples in Fig. 1B, *lanes 6* and *7* were amplified with the 2B2 primers. The mRNA preparations used for the reactions assessed in Fig. 1B, *lanes 6* and *7* were the same as employed for the reactions run in Fig. 1B, *lanes 4* and *5*, respectively. Again, the anticipated size PCR products were produced in each reaction. Higher levels of the 163-bp products were evident when compared with the intensities of the 109-bp bands. However, it was clear that the levels of hepatic PCR product obtained in these experiments did not correlate with the highly induced levels of 2B1/2 mRNA levels present in this tissue (10, 11). These results were interpreted as indicative of saturating input cDNA concentrations in our PCR reactions. To define the dynamic range of our system, we, therefore, conducted 25-cycle PCR reactions with serially diluted cDNA preparations, with the cDNAs being synthesized from PB-induced liver RNAs. The results of this experiment are presented in Fig. 1C.

Each lane of Fig. 1C was loaded, respectively, with one fifth of the total PCR reaction volume (the same proportion as used in the gels of Fig. 1, A and B). The level of product achieved in Fig. 1C, *lane 1* resulted from amplification of cDNA corresponding to 0.5 μg of total cellular RNA (as in Fig. 1, A and B; see Materials and Methods for details of the reaction scheme). Fig. 1C, *lane 2* was loaded with a reaction from the equivalent of 0.05 μg of RNA, representing a 1:10 dilution of the cDNA concentration employed for Fig. 1C, *lane 1*. Fig. 1C, *lane 3* contains a sample from a 1:100 cDNA dilution, *lane 4*, a 1:1000 dilution, and, *lane 5*, a $1:10^6$ dilution. The results demonstrated that the 1:100 cDNA dilutions of PB-induced liver samples were not saturating, because product levels generated were within the dynamic range of the amplification reactions.

Consequently, in subsequent studies we utilized 1:100 cDNA dilutions to more effectively evaluate the relative RNA expression profiles for 2B1 and 2B2. These experiments were conducted with cDNAs reverse-transcribed from hepatic mRNAs isolated from untreated and PB-treated animals at differing stages of development. The PCR data are presented in Fig. 2. The upper portion of each panel illustrates the ethidium bromide-stained PCR reaction products. The lower portions of the panels present data obtained upon Southern transfer and hybridization of the PCR products with internally targeted radiolabeled probes. As negative controls, the last two lanes in each panel contain PCR products resulting from the amplification of RNA samples that had not been subjected to reverse transcription. In each panel, the intensity of autoradiographic signals generated were consistent with the respective fluorescence levels of ethidium bromide-stained gel products. Previously, we could not detect any 2B1 or 2B2 RNA in gestational day 20 rat livers, regardless of pretreatment (15). The present PCR data clearly indicate hepatic expression of both RNAs at this time. Low levels of expression also were noted for the 2B1 and 2B2 mRNAs in the PCR experiments with gestational day 15 livers. In day 15, day 20, and day 46 samples, higher levels of PCR product were present in the PB-induced preparations, when

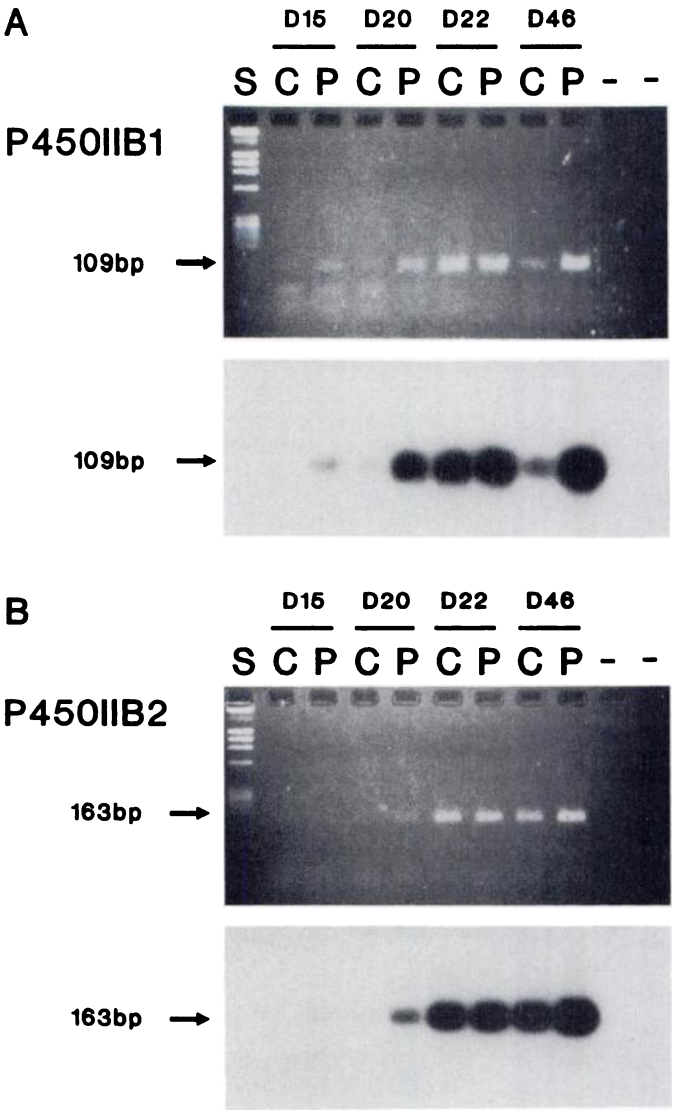

Fig. 2. Rat hepatic CYP2B1 and 2B2 developmental expression profiles determined by PCR amplification. Total RNA was isolated from rat liver at different stages of development and subjected to PCR amplification with specific primer sets. In each *panel*, both ethidium bromide-stained gels (*upper*) and autoradiographic results of Southern blotted membranes (*lower*) are presented. A, Samples amplified with CYP2B1 primer sets; B, 2B2 primers. The ages, in days (e.g., *D15* = day 15), of rats from which the livers were isolated are indicated at the *top* of each *panel*. *S*, molecular size markers; *C*, control, or untreated; *P*, PB-treated; −, negative control samples in which RNAs were amplified directly, without reaction with reverse transcriptase. *Arrows*, the 109-bp (2B1) and 163-bp (2B2) PCR products.

compared with the controls. The preparations from day 22 were anomalous in this respect, because no clear difference was apparent when levels of control and induced PCR products were compared. We recognized previously that both 2B1 and 2B2 mRNA levels were transiently increased in control fetal livers at day 22 in gestation (14, 15).

Fig. 3 presents data generated from a developmental PCR analysis of another strongly PB-inducible P450, CYP3A1. The autoradiographic results obtained following Southern transfer and hybridization of the PCR blots are presented. The fluorescence intensities of the ethidium bromide-stained gels correlated closely with the levels of autoradiographic signal (data not shown). The data illustrated in Fig. 3A were obtained

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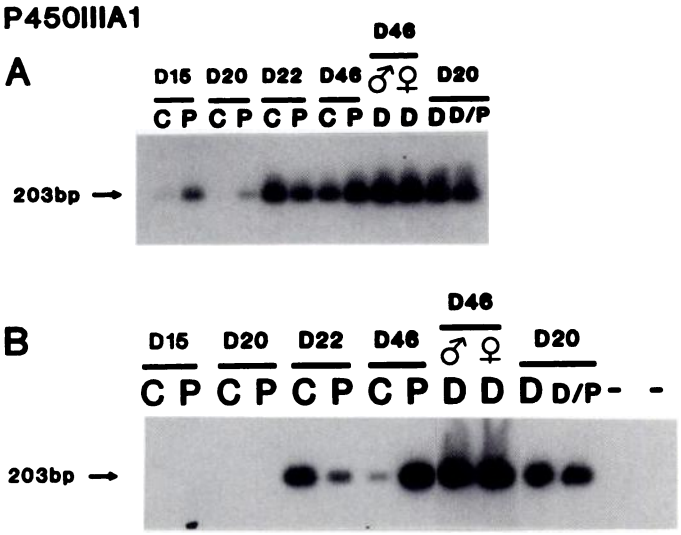

Fig. 3. Developmental expression profile of rat CYP3A1 hepatic mRNAs assessed by PCR. Autoradiographic exposures are shown of Southern blotted PCR product gels after probing with a ^{32}P-labeled 3A1 hybridization oligomer. The abbreviations are as described for Fig. 2, with the addition of dexamethasone-treated samples (*D*) and dexamethasone/PB combined treatments (*D/P*). A, Data from amplification of undiluted liver cDNA preparations; B, data from cDNA fractions diluted 1:100 (see Fig. 1 and text for further explanation of dilution schemes). *Arrows*, the position of the 203-bp 3A1 PCR product.

subsequent to amplification of undiluted cDNA preparations, whereas those in Fig. 3B resulted from PCR reactions conducted with 1:100 cDNA dilutions (as for Fig. 2).

A single 203-bp band was detected in the CYP3A1 experiments, the expected size for the 3A1 amplified product (see Materials and Methods and Table 1 for primers employed). In Fig. 3A a 3A1 PCR product was visible from the gestational day 15 and day 20 livers, and higher levels of product were apparent in the PB-induced samples, compared with controls. The remaining PCR products generated from day 20 through day 46 liver samples were all apparently similar in level. Consequently, we repeated the analysis with 1:100 diluted cDNA preparations and obtained with data included in Fig. 3B. Amplification of the diluted samples produced the expected disproportionate levels of 3A1 mRNA in control versus PB-induced livers. For example, at day 46 (approximately 3 weeks postpartum), much higher levels of PCR signal are demonstrated in PB-treated, as well as in both male and female dexamethasone-treated, livers when compared with the control. Of interest, transplacental treatment with dexamethasone, or a combination of dexamethasone and PB, also resulted in elevated levels of 3A1 PCR product in the day 20 fetal livers, levels that were markedly higher than produced by treatment with PB alone. Although amplification of the diluted cDNAs yielded results consistent with effects of inducers on 3A1 levels measured on older animals with Northern blot analysis (see Fig. 4), the low levels of expression in the day 15 and day 20 livers prohibited detection of PCR product in these experiments (in contrast to the data generated from undiluted cDNAs shown in Fig. 3A). Similar to our results for CYP2B1/2 (Fig. 2), day 22 fetal livers demonstrated comparatively high constitutive levels of 3A1 mRNA, higher than the corresponding level observed in PB-induced tissue of the same age animal.

To illustrate both the enhanced sensitivity of the PCR analyses over that obtainable by Northern blotting and the comparability of results achieved by each technique, a Northern blot analysis of CYP3A1 developmental expression is presented in Fig. 4, *upper*. The same CYP3A1HP oligomer was employed as hybridization probe in both the Northern blotting and PCR experiments. The results of both analyses clearly demonstrated marked induction of 3A1 mRNA by PB treatments. With the exception of day 22, constitutive levels as well as the PB-induced levels of 3A1 mRNA increased with increasing developmental age. The earliest gestational time when 3A1 hepatic mRNA could be detected by Northern blotting was day 19. However, a 5-day autoradiographic exposure was required to detect the very faint signal observed (as opposed to a 18-hr exposure for the remaining panels of the 3A1 blot in Fig. 4, *upper*). In comparison, the PCR data demonstrated that 3A1 mRNA is expressed in the rat liver as early as day 15 of gestation.

Although not assessed by PCR, CYP2C6 mRNA was evaluated by Northern blotting for comparison of the levels and patterns of expression for PB-inducible hepatic P450s, and the data are shown in Fig. 4, *lower*. Very low levels of 2C6 mRNA were observed in the day 29 (1 week postpartum) neonates, but 2C6 mRNA clearly was inducible by PB in older animals (days 46 and 62). No 2C6 mRNA was detected in day 22 or day 19 fetal liver, in control or induced rats.

In situ hybridization experiments were conducted using the livers of day 46 (6 week postpartum) rats, either uninduced or pretreated with PB. The spatial expression patterns were eval-

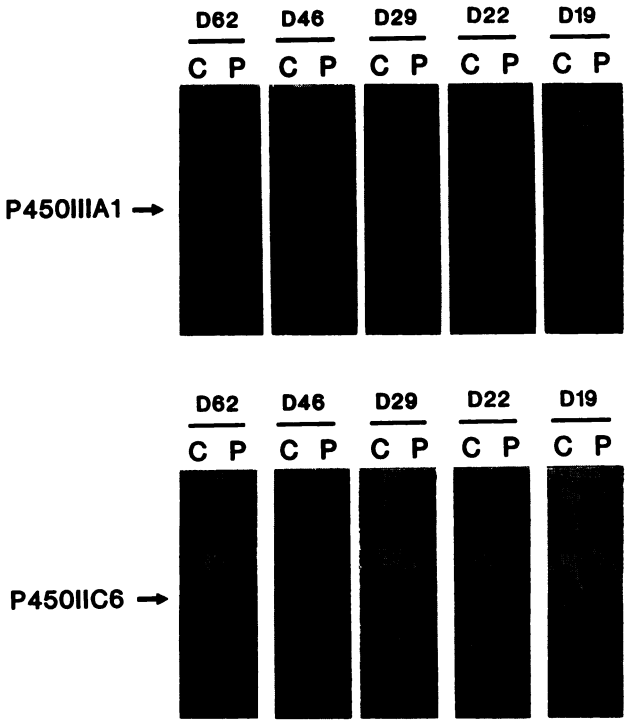

Fig. 4. Northern blot analysis of CYP3A1 and CYP2C6 developmental expression in rat liver. Twenty micrograms of total RNA, isolated from rats at different developmental stages, were applied in each lane and hybridized to the respective 3A1 and 2C6 hybridization probes. Abbreviations are as described for Fig. 2. *Arrows*, the migration positions of the 2.1-kb 3A1 mRNA (*upper*) and 1.8-kb 2C6 mRNA (*lower*). *Upper*, autoradiograph was exposed for 18 hr for all samples except day 19, which was exposed for 5 days. *Lower*, the exposure time was 5 days.

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uated using oligomer probes specific for CYP2C6 and CYP3A1. These data are presented in Fig. 5–7.

Fig. 5 illustrates the data obtained with the CYP2C6 probe in uninduced (Fig. 5, A–C) and PB-pretreated animals (Fig. 5, D–F). In uninduced rats, the grain densities observed appeared to be relatively homogeneous across the hepatic lobule, with no remarkable variation in expression between the centrilobular, midzonal, or periportal regions. Following treatment with PB, there was an increase in the number of grains throughout the lobule, with a slightly decreasing gradient extending away from the central vein toward the portal tracts. In contrast, Fig. 6 presents the results of *in situ* studies conducted with the CYP3A1 probe in control (Fig. 6, A–C) and PB-induced rats (Fig. 6, D–F). Although differences in distribution were not apparent in the untreated liver, in PB-induced liver grain densities were distinctly enhanced in the centrilobular regions, in comparison with the mid-zonal or periportal regions.

Discussion

In this study, we employed PCR amplification, Northern blotting, and *in situ* hybridization techniques to evaluate PB-

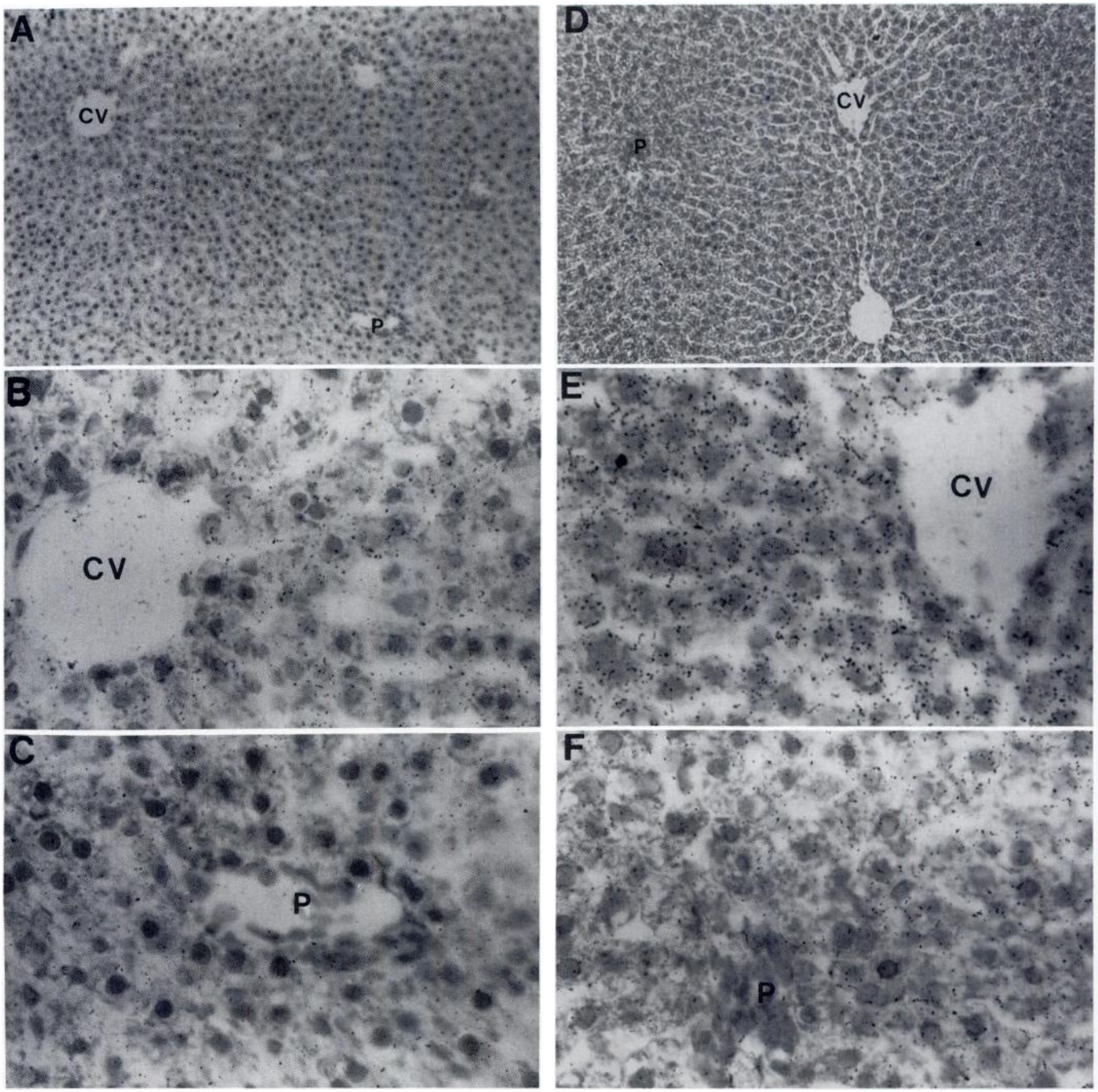

Fig. 5. *In situ* hybridization analysis of hepatic CYP2C6 mRNA expression in control and PB-treated rats. Central veins (*CV*) and periportal regions (*P*) are indicated in each *panel*. A–C, From control animals; D–F, from rats pretreated with PB. A and D, 100× magnification fields from which the higher magnification photos (400×) of B and C, and E and F, respectively, were taken. Autoradiographic exposure time for the 2C6 experiments was 4 weeks.

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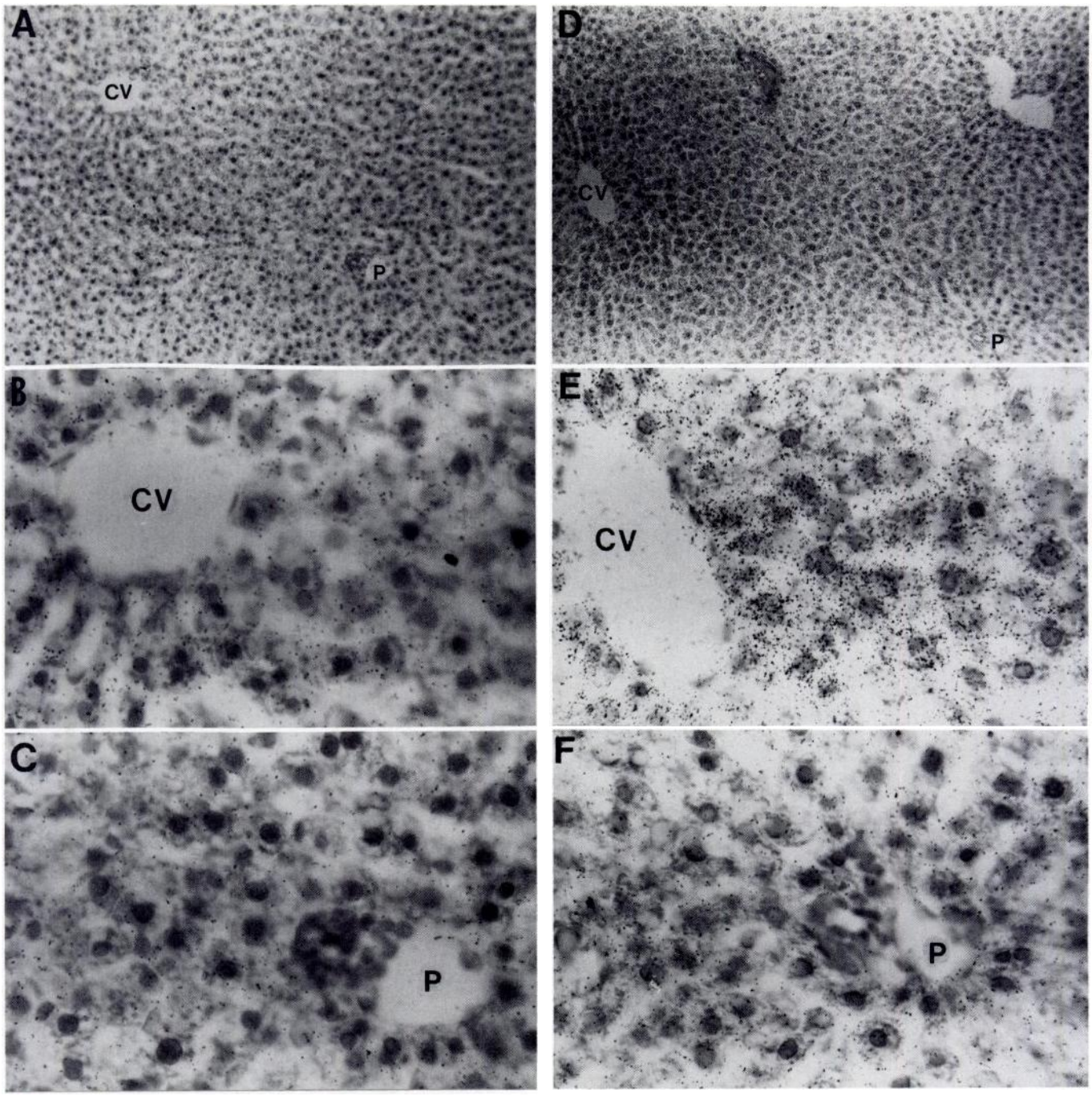

Fig. 6. *In situ* hybridization analysis of hepatic CYP3A1 mRNA expression in control and PB-treated rats. Central veins (*CV*) and periportal structures (*P*) are designated in each *panel*. A–C, From control animals, D–F, from rats pretreated with PB, as in Fig. 5. A and D, full field, 100× magnification photos from which the higher magnification photos (400×) of B and C, and E and F, respectively, were obtained. Autoradiographic exposure time was 3 weeks.

inducible P450s. We demonstrated that the PCR approach provides a highly sensitive assay allowing discriminative analysis of P450 mRNAs in tissues where mRNAs were not readily detectable with other techniques. The procedure also proved especially useful in assessing P450 expression in liver tissues obtained from early developing rats. This is the first report detailing the use of PCR technology to examine mRNA expression levels of PB-inducible genes during rat development.

Previously, with standard blotting and solution hybridization assays, we were not able to detect CYP2B1 or 2B2 mRNAs in PB-inducible fetal rat livers before gestational day 21 (15). With the PCR data presented here (Fig. 2), both mRNAs were shown to be expressed by day 15 of gestation (the earliest day in development at which we were able to macroscopically recognize and dissect the fetal liver). Although the levels of expression in the day 15 fetuses were substantially less than at later stages of development, both mRNAs clearly were inducible by PB pretreatments at this early age. Similar conclusions regarding the early onset of PB responsiveness were drawn from our experiments examining the developmental expression of CYP3A1 mRNAs.

Transplacental treatment of pregnant rats with PB also

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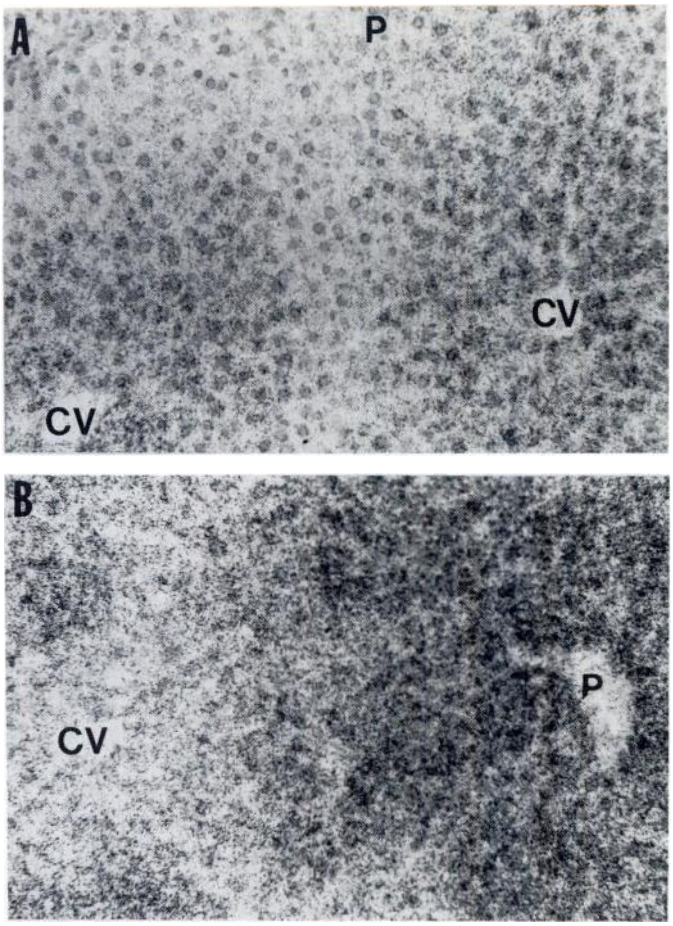

Fig. 7. *In situ* hybridization analysis of hepatic CYP3A1 and serum albumin mRNAs in PB-induced rats. A, The predominant centrilobular (*CV*) expression of 3A1 in the hepatic lobule; B, the preferential periportal (*P*) localization of serum albumin mRNA. The magnification in each panel was 160×. Exposure times were 3 weeks.

resulted in increased levels of 3A1 mRNA in day 15 fetal livers (Fig. 3). In comparison, even after lengthy autoradiographic exposure of Northern blots, only low levels of CYP3A1 mRNA were detected in day 19 PB-treated fetal liver, and no expression was apparent in day 15 liver (Fig. 4). Analysis of 3A1 mRNA levels in PB-treated fetal rats has not been reported previously. In a previous report, CYP3A protein levels have been assessed in fetal rat liver by immunoassay (23). In agreement with the results reported here, induced levels of fetal hepatic 3A protein also were detected in that study; however, in contrast to the PCR data, no 3A1 protein was detectable by immunoblotting in untreated fetal liver at any time during gestation (23). These comparative data illustrate further the enhanced sensitivity achieved by the PCR-based method.

Hepatocytes appear to be unique, relative to most other cell types [one exception is intestinal enterocytes, where the CYP2B1 gene also has been reported to be PB responsive (24)], with respect to their capacity to be activated transcriptionally by PB and related inducing substances (7, 11). The data presented here demonstrate that multiple genetic loci possess the potential for PB activation early in liver organogenesis and that the responsiveness intensifies with increasing developmental age. However, the identity of the liver-specific factor(s) and/or *cis*-regulatory sequences responsible for PB induction in this tissue remains obscure. Several transcriptional regulatory factors have been identified in liver extracts, including a liver-specific factor known as HNF-1 (25), which is most likely equivalent to APF (26), LF-B1 (27), and HP1 (28). A sequence homologous to the recognition site of HNF-1 is present within the promotor regions of several liver-specific genes, including albumin, α-1-antitrypsin, β-fibrinogen, and α-fetoprotein (25). Of interest with respect to the data reported here, the HNF-1 factor appears to be developmentally regulated, such that in rats transcription is first detected on day 14 of gestation and increases more than 20-fold by day 20 of gestation (29). The possibility that HNF-1 participates in the regulation of CYP2B1/2 and 3A1 gene expression is, therefore, intriguing. However, computer analysis of 5′-flanking sequences for the rat 2B1 and 2B2 genes (through base −1650) did not result in a clear candidate for a HNF-1 recognition sequence. The most closely related 2B1/2B2 gene sequences possessed only 13 bases in common with the 21-base elements identified as HNF-1 binding domains for either the rat serum albumin or α-fetoprotein genes (25).[2]

Although the liver is certainly a PB-responsive organ, the question presents itself as to whether certain hepatocytes are differentially activated by PB within the liver lobule. To this end we examined previously the distribution of CYP2B1 and 2B2 mRNAs across the hepatic lobule with *in situ* hybridization analysis (18). The results of those experiments demonstrated that the PB-induced pattern of expression for 2B1 versus 2B2 was indistinguishable. Only a small band of cells, immediately surrounding the periportal tract, were refractory to induced levels of 2B1 and 2B2 expression. The remaining centrilobular and mid-zonal regions exhibited uniform and elevated levels of grain density in PB-induced animals (18). Consequently, we hypothesized that the factor(s) necessary for promoting genetic responsiveness to PB indeed are expressed in those hepatocytes reflecting this particular zonal distribution. Therefore, we reasoned that it might be expected that other PB-inducible genes would exhibit similar patterns of spatial expression across the liver lobule.

To examine this issue, we conducted *in situ* hybridization studies to localize the mRNAs for two other PB-inducible P450 genes, 3A1 and 2C6. The experiments were conducted with serial sections of the same livers used for the 2B1 and 2B2 experiments (18). These data indicated clearly that 3A1 and 2C6 exhibited unique patterns of distribution in the hepatic lobule, unique both in comparison with those observed for 2B1 and 2B2 and with respect to each other. Results presented in Figs. 6 and 7 clearly indicate a predominant centrilobular expression pattern of 3A1 in PB-induced liver. In contrast, the 2C6 expression profile (Fig. 5) was much more uniform across the lobule, somewhat similar to that observed for 2B1/2, with the exception that PB-induced grain density was lower and the layer of periportal cells refractory with respect to 2B1/2 expression were not refractory to 2C6 expression.

The distinctive centrilobular expression patterns of CYP3A1 are further demonstrated in the autoradiographic exposure shown in Fig. 7A. For comparison with the 3A1 results, we also conducted *in situ* hybridization analyses with a probe for rat albumin. These data are shown in Fig. 7B. From the resulting grain distributions, it is clear that albumin mRNA exhibits the

[2] Ramsden, R., Omiecinsk, C. J., Unpublished observation.

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converse regional profile, demonstrating primarily periportal expression.

These observations regarding PB induction profiles in the liver lobule are intriguing. In one sense, it appears that almost all hepatocytes, regardless of their zonal distribution within the liver lobule, possess the core regulatory factor(s) necessary for PB responsiveness. However, because distinctive regional patterns of expression are noted for individual PB-responsive genes, it appears either that 1) individual PB-responsive genes harbor PB-regulatory sequences that are unique to each gene and provide differing potentials for interaction with key regulatory proteins or that 2) the molecular processes conferring PB responsiveness are redundant, such that different single, liver-specific factors can each individually promote the gene induction process or that multiple combinations of individual factors can interplay to activate gene transcriptional events. Differentiation between these or other possible explanations for the distinctive regional expression profiles of PB-inducible genes in the liver will require the identification and characterization of the molecular factors critical to the induction response.

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Send reprint requests to: Curtis J. Omiecinski, Ph.D., Department of Environmental Health, SC-34, University of Washington, Seattle, WA 98195.

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