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Developmental Regulation of Cytochrome P-450 Genes in the Rat

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SUMMARY

Synthetic oligomer probes were used in hybridization experiments to investigate the developmental regulation of cytochrome P-450 (P-450) genes in rat liver. Transplacental induction by phenobarbital of P-450b and P-450e mRNAs was not detectable in fetal rat livers prior to day 21 of gestation. The levels of these mRNAs increased approximately 2-fold from gestational day 21 to day 22 in phenobarbital-induced liver. P-450b and P-450e mRNAs were shown to be adenylated and the fractions associated with polysomes were similar in both fetal and adult livers. No P-450b or P-450e mRNAs were detected in fetal lung and kidney RNA preparations regardless of pretreatment. Southern blot data utilizing fetal liver DNA suggests that responsiveness to xenobiotic induction during development is not attained by rearrangement of P-450b or P-450e genes. Experiments with probes specific for P-450c and P-450d failed to detect their respective mRNAs in fetal livers from 3-methylcholanthrene (3-MC)-treated or untreated rats. Both species were detectable in 3-MC-treated rats 1 week after birth. The levels of 3-MC-inducible P-450c and P-450d mRNAs increased with age and peaked approximately 3 weeks after birth. Hepatic P-450d mRNA levels in 3-MC-treated or control rats was consistently higher than P-450c mRNA levels at all ages studied.

Mammalian P-450s are hemoproteins enriched in the smooth endoplasmic reticulum that are responsible for a majority of phase 1 biotransformation reactions with xenobiotic and endogenous substrates (reviewed in Ref. 1). Although early studies suggested that P-450 activities were nearly absent in embryonic and fetal tissue (reviewed in Ref. 2), more recent studies have shown that these enzymes may be activated even during early development. Filler and Lew (3) demonstrated that late preimplantation mouse embryos were able to metabolize benzo[*a*]pyrene, most likely via P-450-mediated reactions. Similarly, Juchau *et al.* (4) showed that day 10 rat embryos induced *in utero* with 3-MC could metabolize 2-acetylaminofluorene to teratogenic intermediates, and that this metabolism could be inhibited by culturing the embryos in an atmosphere containing carbon monoxide, a prototypical P-450 inhibitor (5). 3-MC also induced P-450$_1$ mRNA accumulation in mouse livers as early as day 15 of gestation (6). Thus, it appears that 3-MC-inducible P-450 systems may be active during the highly sensitive developmental stages of an organism. Since the P-450s are capable of bioactivating as well as detoxifying xenobiotics, it is critical to understand the factors influencing the regulation of these enzymes.

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Recently, evidence for the existence of PB-inducible P-450s in developing rodent embryos was presented. Oligodeoxynucleotide probes specific for P-450b and P-450e developed in our laboratory (7) were used to assess P-450b and P-450e hepatic mRNA accumulation in rat fetuses during ontogeny (8). In that study we found that both mRNAs were PB-inducible at 22 days of gestation but not on day 19 or at earlier stages of development. The inducible levels of P-450b and P-450e mRNAs increased as a function of age, peaking approximately 3 weeks after birth. Employing immunological techniques, Cresteil *et al.* (9) detected PB-B (P-450b+e) in day 22 rat fetuses transplacentally exposed to PB. Only combined levels of P-450b and P-450e were measured, however, as these cytochromes are immunologically identical (10) and exhibit extraordinary homologies in their primary structures (11) and substrate specificities (12).

The following experiments were undertaken to better define the expression of P-450 isozymes during development. The exact onset of P-450b and P-450e mRNA inducibility by PB in the developing rat was assessed along with characterization of the mRNA products with respect to adenylation status and compartmentalization within fetal liver cells. The levels of these mRNAs were also studied in fetal extrahepatic tissues. Genomic DNA restriction analyses indicated that no obvious changes in P-450b or P-450e gene organization occurred during

ABBREVIATIONS: P-450, cytochrome P-450; 3-MC, 3-methylcholanthrene; PB, phenobarbital; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; kb, kilobase.

development. In this paper we also describe and characterize two newly developed oligomer probes for the 3-MC-inducible isozymes, P-450c and P-450d. Studies utilizing these probes to measure their mRNA levels in control and 3-MC-treated rat livers at various ages in development are presented.

Materials and Methods

Materials were obtained as follows: 3-MC (98%) from Aldrich Chemical Co. (Milwaukee, WI); [^{32}P]dATP, dCTP, dGTP, and dTTP (800 Ci/mmol) from New England Nuclear Research Products (Boston, MA); pd(N)$_6$ from Pharmacia (Piscataway, NJ); HEPES from Sigma Chemical Co. (St. Louis, MO); and Minifold 11 Slot Blotter from Schleicher and Schuell (Keene, NH). Other materials were purchased from sources previously described (7, 8, 13).

Animal treatment. Sprague-Dawley rats were obtained from Tyler Labs, Inc. (Bellevue, WA). To assess PB induction, adult, pregnant and immature rats were injected intraperitoneally with either 50 mg/kg PB or saline alone (*controls*) for 3 consecutive days, the last dose given 16 hr before killing. For 3-MC induction studies, animals received 40 mg/kg 3-MC dissolved in corn oil by intraperitoneal injection for 2 consecutive days before sacrifice. Previous studies have shown that saline-treated animals display P-450 patterns identical to those treated with corn oil alone.[1] The rats were housed in plastic cages and given access to food and water *ad libitum*. A 12-hr light/dark cycle was maintained. The day following timed mating was considered day 0 of development. The period of rat gestation is 22 days.

RNA and DNA preparation. Livers from at least three rats were dissected and pooled for each age and treatment group. To assess the prenatal time points, 10–30 fetuses obtained from at least two pregnant rats were pooled for RNA and DNA isolation. Total rat liver RNA was prepared exactly as described (8). polyA$^+$ and polyA$^-$ RNAs were separated from total RNA by two rounds of oligo dT-cellulose chromatography. Polysomal RNA was isolated by $MgCl_2$ precipitation as follows. Tissue was homogenized in 10 volumes of buffer A (25 mM Tris, pH 7.5, 25 mM NaCl, 5 mM $MgCl_2$, 1 mg/ml heparin sulfate, 2% Triton X-100). The homogenate was centrifuged at 15,000 rpm for 1 min at 0°. The supernatants were immediately transferred to tubes containing an equal volume of cold buffer B (4 volumes of buffer A, 1 volume of 0.1 M $MgCl_2$). Polysomes were precipitated by incubation for 60 min at 4°. The samples were then layered over sucrose cushions (1 M sucrose in 25 mM Tris, pH 7.5, 25 mM NaCl, 5 mM $MgCl_2$) and centrifuged at 12,000 rpm for 10 min at 4°. The supernatants were discarded and the polysomal pellets were dissolved in 0.1 M NaCl, 10 mM Tris, pH 7.5, and 50 mM EDTA. SDS was added to a final concentration of 1% and the samples were extracted with an equal volume of phenol followed by two extractions with PCI (49% phenol/49% chloroform/2% isoamyl alcohol). Polysomal RNA was ethanol precipitated, washed with 70% ethanol, and resuspended in sterile water for use in the experiments presented.

DNA was prepared using the method of Blin and Stafford (14).

DNA probes. The oligomers used to assess P-450b and P-450e mRNA levels have been previously described (7). The oligomers used to assess P-450c and P-450d mRNA levels were synthesized with an Applied Biosystems DNA synthesizer in the Solid Phase Synthesis Center of the Howard Hughes Medical Institute at the University of Washington. For use as probes, 5′-end labeling was performed as described (7, 8). The resulting specific activities were typically $\geq 9 \times 10^6$ cpm/pmol.

The cDNA probes utilized in this study were pRSA57, a rat liver albumin cDNA previously described (15), and the 400-base pair *Pst* 1 fragment of clone 46, a mouse P$_1$450 cDNA clone (16) which has been shown to cross-react with rat liver P-450c mRNA (17). For use as probes, the cDNAs were radiolabled according to the primer extension technique of Feinberg and Vogelstein (18, 19) with the exception that 1.9 μmol each of [^{32}P]dATP, dCTP, dGTP, and dTTP were used in the reaction with no additional unlabeled nucleotides. In this way we obtained 40–50% incorporation of label into the cDNA probes and specific activities of 1×10^9 cpm/μg.

Solution hybridization experiments. Solution hybridization experiments were performed exactly as described (7).

RNA blot experiments. Northern blotting of total RNA was performed exactly as described (13). Slot blotting of RNA onto GeneScreen Plus (New England Nuclear) membranes was performed according to the manufacturer's guidelines. Hybridizations using oligomers were performed as previously reported (8) except that the final wash was performed at 52° in 5 × SSC/0.1% SDS (1 × SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0).

Membranes for cDNA hybridization were prehybridized for 2 hr and hybridized overnight at 42° in buffer containing 50% formamide, 0.75 M NaCl, 50 mM Tris, pH 7.5, 1× Denhardt's (0.02% Ficoll, 0.02% polyvinylpyrrolidone 360, 0.02% bovine serum albumin), 1% SDS, 10% dextran sulfate, 200 μg/ml denatured salmon sperm DNA, and $2–4 \times 10^5$ cpm/ml [^{32}P]cDNA. GeneScreen Plus filters were washed exactly as described by the manufacturer.

Autoradiography was performed for varying lengths of time at −70° using Kodak XAR-5 film with DuPont Lightning Plus intensifying screens. Molecular sizes were estimated by comparison of bands to ^{35}S-labeled DNA fragments of λ phage generated by restriction endonuclease digestion with *Hind* 111 that were run on gels concurrently.

Southern blot experiments. Southern blot analysis was performed as follows: 15 μg of DNA isolated from fetal rats were restricted with 50 units each of either *Eco R*1, *Hind* 111, or *Pst* 1. Following phenol extraction and ethanol precipitation, the resulting fragments were electrophoresed at 30 V for 16 hr in 0.85% agarose gels containing 1.0 μg/ml ethidium bromide. The gel was soaked for 30 min in transfer buffer (0.4 M NaOH, 0.6 M NaCl) and then blotted overnight onto a GeneScreen Plus membrane with fresh transfer buffer. The filter was neutralized by soaking 15 min in a solution containing 0.5 M Tris, pH 7.0/1 M NaCl. The filter was air dried and then used for hybridizations with either P-450b or P-450e oligomer. ^{32}P-Oligomer (1×10^6 cpm/ml) was hybridized to the membrane overnight at 37° in a solution containing 50% formamide/5× SSC/25 M $NaPO_4$, pH 6.5/1% SDS/1× Denhardts/10 μg/ml polyadenylic acid. Membranes were washed twice at room temperature for 15 min each with 5× SSC/0.1% SDS, twice at 37° for 5 min each with TMA solution (3 M tetramethylammonium chloride/50 mM Tris-HCl, pH 8.0/2 mM EDTA/1% SDS), and twice at 53° for 30 min each with TMA solution. The final washes correspond to conditions that allow only exact 18 base match DNA:DNA hybrids to form (20).

Results

Hybridization analyses of P-450b and P-450e mRNA levels. The onset of PB responsiveness in the developing rat was determined by Northern blot analysis of total hepatic RNA utilizing P-450b and P-450e oligomers. The data in Fig. 1A demonstrate that PB treatment significantly elevated P-450b mRNA levels above control levels beginning 21 days after conception (Fig. 1A, *lanes 5* and *6*). PB induction was not evident on gestational day 19 (Fig. 1A, *lanes 1* and *2*) or 20 (Fig. 1A, *lanes 3* and *4*). A similar developmental profile was observed using the P-450e oligomer (Fig. 1B). Levels of PB-inducible P-450b and P-450e mRNAs increased with age. Solution hybridization experiments (Table 1) showed that the levels of P-450b mRNA in PB-treated day 21 fetal livers were 0.6 times the day 22 levels. Similarly, there was 0.5 times the amount of P-450e RNA in day 21 fetal livers compared to day 22 livers. Significant levels of both P-450b and P-450e mRNA were observed in control day 22 neonates (Fig. 1, A and B, *lane 7*). The molecular size of the mRNAs observed for both P-450b

[1] Unpublished observation.

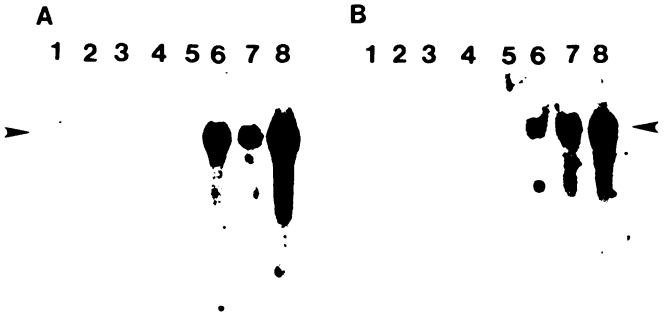

Fig. 1. Northern hybridization analysis of hepatic P-450b and P-450e mRNAs in fetal rats. Twenty μg of total hepatic RNA isolated from day 19 (*lanes 1* and *2*), day 20 (*lanes 3* and 4), day 21 (*lanes 5* and *6*), and day 22 (*lanes 7* and *8*), control (*lanes 1, 3, 5,* and *7*) or PB-treated (*lanes 2, 4, 5,* and *8*) rats were subjected to Northern blot analysis. Filters were hybridized to [^{32}P]-P-450b (A) and [^{32}P]-P-450e (B) oligomers. The *arrowhead* indicated the position of migration of P-450b and P-450e mRNAs (1.8 kb).

TABLE 1

Quantitation of mRNA concentrations for cytochrome P-450b and P-450e in PB-treated rat liver

Data were derived from the average of three experiments, each performed in triplicate, with <15% variation between average values. P-450b and P-450e total (tot) and polysomal (poly) mRNAs were below the detection limits in Pb-treated day 19 and day 20 rats. Our calculations assume that polysomal RNA comprises 80% of the total hepatic RNA (34).

Age[b]	pg P-450b mRNA/μg tot or poly RNA[a]			pg P-450e mRNA/μg tot or poly RNA		
	tot	poly	%[c]	tot	poly	%
21	2.6	2.0	62	0.1	ND[d]	
22	4.3	3.2	60	0.2	ND	
62	31.7	28.3	71	4.9	4.3	70

[a] One to 20 μg of total or polysomal hepatic RNA were hybridized to 4000 cpm of the respective ^{32}P-oligomer as described under Materials and Methods. Only concentrations in the linear range of the observed binding curves were used to calculate concentrations of mRNA. Calculations were made as previously described (7).

[b] Age of rats is given in days of development (the day after timed mating is considered day 0 of development).

[c] [(pg mRNA/μg polysomal RNA)/(pg mRNA/μg total RNA) × 0.8] × 100.

[d] ND, not detectable within the sensitivity limits of this assay (<0.1 pg of mRNA/μg of total RNA).

and P-450e was 1.8 kb, in agreement with previous results (7, 8).

To confirm the authenticity of the data obtained with the oligomer probes, pRSA57, a rat liver albumin probe, was used to probe total rat liver RNA samples. Fig. 2 shows the slot blot analysis of fetal rat liver total RNA probed with [^{32}P]-P-450b (A) and [^{32}P]-pRSA57 (B). The observed developmental profile of albumin mRNA expression (see also Fig. 6C) agrees well with that published by Muglia and Locker (21) and, furthermore, does not exhibit the PB induction phenomenon.

Total mRNA was also isolated from control and PB-treated fetal (day 22) rat lung and kidney. Results of Northern blot analysis (data not presented) indicated that neither P-450b nor P-450e mRNAs were detectable in these tissues.

Since previous studies indicated a difference in the affinities of P-450b mRNAs from various tissues to oligo dT-cellulose (22), it was of interest to assess this parameter in the fetal mRNA isolates. Fig. 3 shows the results of Northern blot analysis utilizing these preparations. Both P-450b (Fig. 3A) and P-450e (Fig. 3B) mRNAs are enriched in the polyA$^+$ fraction of cellular RNA, similar to the adult liver mRNAs.

Membrane-bound and free polysomes were collectively precipitated by $MgCl_2$, and the associated mRNAs were isolated. The results shown in Table 1 demonstrate that approximately 60% of the total P-450b mRNA in fetal samples (days 21 and 22) is bound to polysomes. The levels of total P-450e mRNA were close to the limit of detection of the solution hybridization assay and the polysome-associated fraction could not be quantified with confidence. However, prolonged exposure of slot blot hybridizations (data not presented) indicated a similar association of the fetal P-450e mRNA with isolated polysomes. In the adult liver (day 62 of development), essentially the same proportion (60–70%) of the total P-450b and P-450e mRNAs were bound to the polysomes (Table 1).

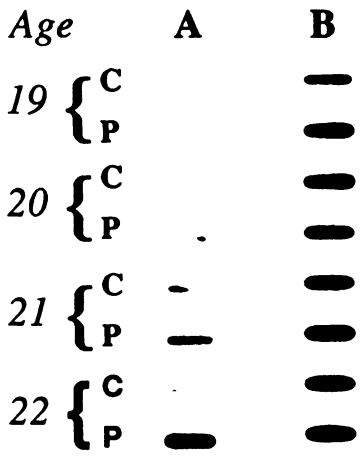

Fig. 2. Slot blot analysis of fetal rat hepatic total RNA. Ten μg of total RNA isolated from control (*C*) or PB-treated (*P*) rats at gestational ages 19, 20, 21, and 22 were slot blotted onto a GeneScreen Plus membrane. The filter was first probed with [^{32}P]-P-450b (A), then dehybridized and reprobed with [^{32}P]-pRSA57 (B).

In a previous paper (8), we noted that PB-induced P-450b and P-450e levels peaked approximately 3 weeks after birth (day 46) and declined in adult (day 62) rats. In those studies only RNA isolated from male rat livers was assessed; therefore, it was of interest to further analyze PB-inducible P-450 development with regard to sex dependence. To do this we performed Northern blot analysis on total rat liver RNA isolated from PB-induced male and female rats. The results are presented in Fig. 4. Both P-450b (Fig. 4A) and P-450e (Fig. 4B) showed sex-specific expression in immature rats. Levels of both mRNAs were higher in PB-treated day 46 male rats (Fig. 4, *lane 6*) compared to the adult levels (Fig. 4, *lane 8*). In contrast, P-450b and P-450e mRNA levels were slightly lower in PB-induced day 46 female rats (Fig. 4, *lane 2*) compared to day 62 adult female rats (Fig. 4, *lane 4*). Identical results were obtained after repeating these experiments, in each case using pooled livers from an additional three male or female day 46, PB-treated rats. No male/female differences were observed in RNA isolated from control rats.

Hybridization analyses of P-450c and P-450d mRNA levels. Oligomeric probes were also utilized in hybridization studies to assess P-450c and P-450d mRNA expression in the developing rat liver. For P-450c, a 20-mer with sequence 5′-d(TCTGGTGAGCATCCAGGACA)-3 was synthesized complementary to the published mRNA sequence (23) from bases 1650 to 1669. For P-450d, a 20′-mer with sequence 5′-d(GGAAAAGGAACAAGGGTGGC)-3′ was synthesized com-

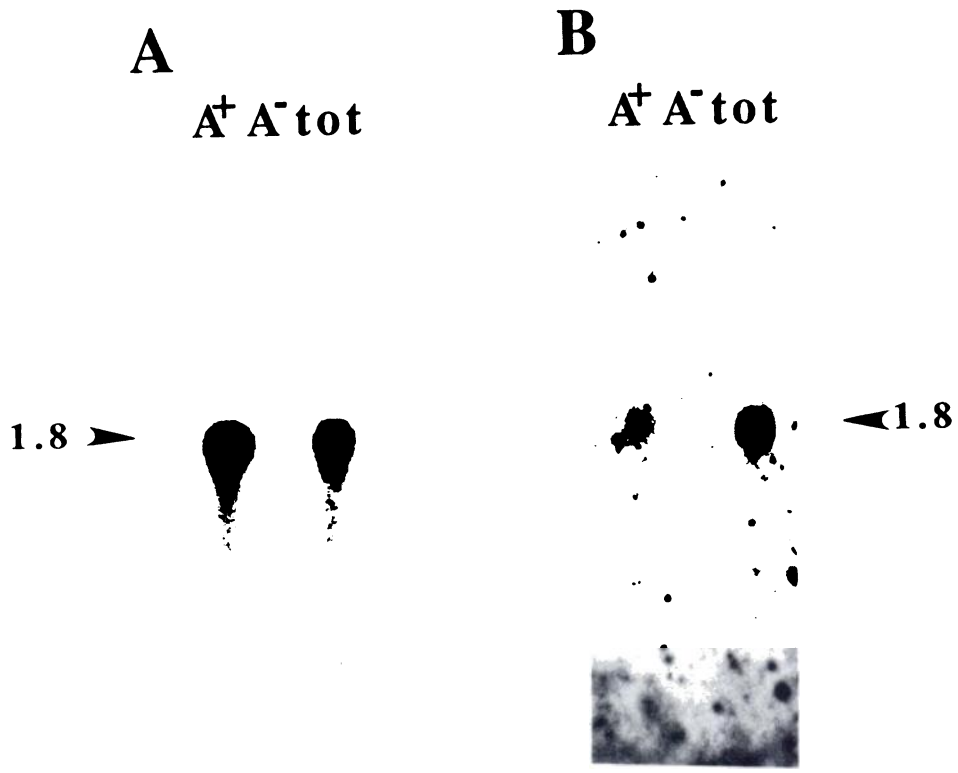

Fig. 3. Adenylation status of fetal P-450b and P-450e mRNAs. One μg of polyA$^+$ RNA (A^+), 20 μg of polyA$^-$ RNA (A^-), and 20 μg of total RNA (*tot*) were subjected to Northern blot analysis. Filters were probed with [^{32}P]-P-450b (A) or [^{32}P]-P-450e (B) oligomers. Molecular size is indicated by *arrowheads*.

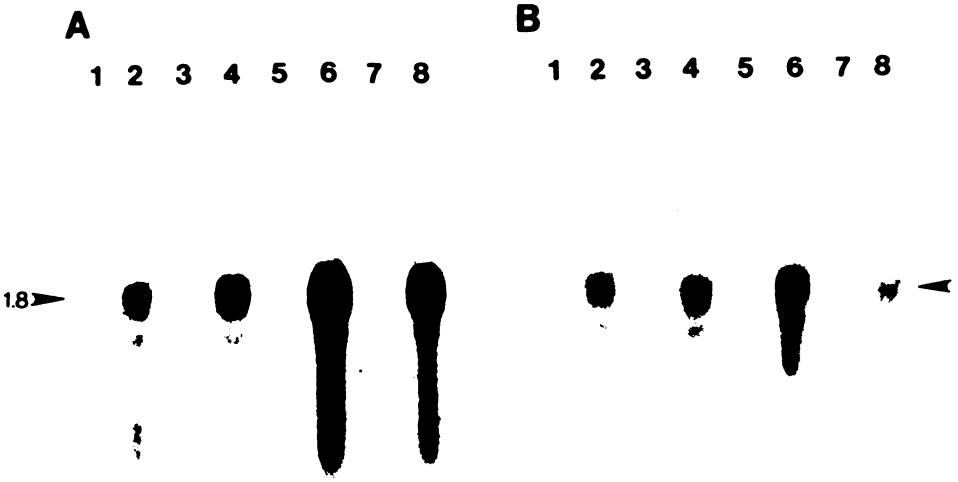

Fig. 4. Sex-dependent expression of P-450b and P-450e mRNA during development. Ten μg of total hepatic RNA isolated from day 46 female rats (*lanes 1* and *2*), day 62 female rats (*lanes 3* and *4*), day 46 male rats (*lanes 5* and *6*), and day 62 male rats (*lanes 7* and *8*) after no treatment (*lanes 1, 3, 5,* and *7*) or PB treatment (*lanes 2, 4, 6,* and *8*) were subjected to Northern blot analysis utilizing [^{32}P]-P-450b (A) or [^{32}P]-P-450e (B) oligomers. Molecular size is given in kilobases.

plementary to the published mRNA sequence (24) from bases 1563 to 1582. Fig. 5 illustrates the results of Northern blot analysis utilizing (A) [^{32}P]-P-450c (Fig. 5A) and [^{32}P]-P-450d (Fig. 5B) oligomers to determine probe specificity. Ten μg of total rat liver RNA from day 46 control (C), 3-MC-induced (M), and PB-induced (P) rats was applied to each lane. The results show that the P-450c probe hybridized exclusively to a 3-MC-inducible mRNA species with molecular size of 2.7 kb. No P-450c mRNA was detectable in control animals at any age examined. In contrast, the P-450d oligomer recognized a 3-MC-inducible mRNA having molecular size of 1.9 kb which could be detected at low levels in control and PB-treated animals. No cross-hybridization of the probes was observed even after prolonged autoradiography.

Fig. 6 shows data obtained from Northern blot analysis of total rat liver RNA at various gestational ages using [^{32}P]-P-450c (Fig. 6A), [^{32}P]-P-450d (Fig. 6B), and [^{32}P]-pRSA57 (Fig. 6C) probes. For both P-450c and P-450d mRNAs, 3-MC inducibility appears to a significant degree on day 29 of gestation (1 week after birth) with levels peaking on day 46 (approximately 3 weeks after birth). The mRNA sizes for both P-450c and P-450d are as expected (see above) and do not appear to change

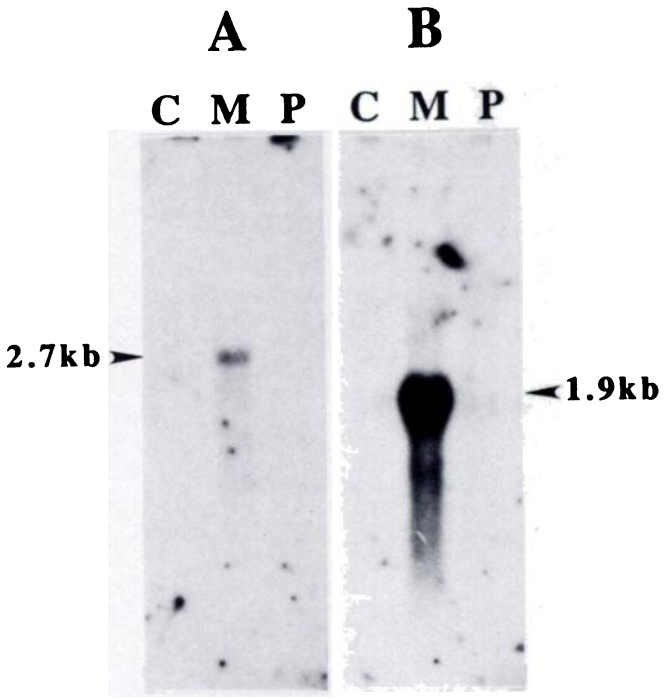

Fig. 5. Specificity of P-450c and P-450d oligomers. Twenty μg of total RNA from control (*C*), 3-MC-treated (*M*), and PB-treated (*P*) day 46 rats were subjected to Northern blot analysis using [^{32}P]-P450c (A) and [^{32}P]-P450d (B) as probes. Molecular size is indicated in kilobases.

with age. PB had no effect on mRNA levels of either species (data not shown). These data were corroborated by reprobing the filters with clone 46, the mouse $P_1$450 cDNA clone (16) and 16′-mer oligomers complementary to different regions of the P-450c and P-450 d mRNAs. Identical results were obtained with each P-450c or P-450d probe in hybridization experiments utilizing the same filters (data not shown). Reprobing of the filters with pRSA57 gave the expected albumin mRNA developmental pattern, which was unresponsive to 3-MC treatment.

Genomic blot analyses of fetal DNA. To investigate the possible mechanisms whereby xenobiotic inducibility is acquired during development, we performed genomic blot analyses with hepatic DNAs isolated from control and PB-treated day 19 and day 21 rat livers. The results are shown in Fig. 7. Four major restriction bands are observed in each lane in the blot probed with [^{32}P]-P-450b and one to two major bands are seen using [^{32}P]-P-450e. The hybridization patterns are identical at both ages independent of pretreatment.

Discussion

The phenomenon of PB inducibility in the rat fetus has been controversial. Early studies of P-450 content and monooxygenase activities suggested that no PB induction occurred prior to birth (25, 26). However, results from our laboratory using Northern blot and solution hybridization studies established that P-450b and P-450e hepatic mRNAs were PB-inducible in day 22 rat fetuses, but not in day 19 or younger fetuses (8). In those studies, however, day 20 and day 21 fetal RNA preparations were not assessed. The current study has extended the previous results and shown that PB responsiveness is first evident in rats on day 21 of gestation. The findings that the mRNAs are adenylated and are associated with the polysomal RNA fraction strongly suggest that these mRNAs are actively utilized by the fetal rat liver. It is likely that the fetal mRNAs detected with the P-450b and P-450e oligomers code for proteins which are identical to those observed in the adult. Recent studies by Cresteil *et al.* (9) have demonstrated the presence in fetal rat liver of a PB-inducible protein, immunochemically and electrophoretically identical to PB-B, whose presence correlated with enhanced benzphetamine-*n*-demethylase and benzo[*a*]pyrene hydroxylase activities. Taken together, these data, obtained utilizing three independent techniques for measuring P-450 gene expression, clearly indicate that transplacental PB treatment stimulates cytochrome P-450 expression in the developing rat. These findings underscore the importance of considering fetal biotransformation capabilities when assessing potential chemical toxicity.

In contrast to the findings in fetal rat liver, no P-450b or P-450e mRNAs were detected in control or PB-induced fetal lung and kidney RNA isolates. Previous studies in our laboratory (22) have shown that neither of the P-450 mRNAs are detectable in the adult rat kidney, but that P-450b mRNA does accumulate in the lung independent of pretreatment at a level of approximately 10% that seen in the PB-induced adult liver. P-450e mRNA was not detected in adult lung preparations. These findings indicate that P-450b and P-450e expression in extrahepatic tissues further differ from their liver counterparts with regard to developmental time course.

Results of genomic blot hybridizations performed using the P-450b and P-450e oligomer probes indicate that potentially four P-450b-like genes and two P-450e-like genes exist. Similar findings indicating multiple P-450b- and P-450e-like genes in rat liver have been reported (27, 28). The restriction patterns observed utilizing the P-450b and P-450e probes were identical for day 21 (PB-responsive) and day 19 (PB-unresponsive) fetal DNAs, regardless of pretreatment. These data indicate that no gross gene rearrangements are coincident with the onset of PB inducibility within the P-450b or P-450e genes. How, then, is the ability to respond to inducing agents during P-450 development regulated? One possibility is that a necessary receptor or cofactor required for PB-induction does not appear in the rat liver until day 21 of gestation. Since a receptor for PB has not been found, however, it is difficult to address this possibility. As actively transcribed genes share, in general, certain characteristics including modified chromatin structures and methylation differences compared to nontranscribed genes, we are currently attempting to assess these parameters in the fetal P-450b and P-450e genes.

In this paper we also present studies utilizing oligomeric probes for P-450c and P-450d, the rat analogues of mouse $P_1$450 and $P_3$450, respectively. Northern blot analysis showed that the P-450c oligomer recognized a 3-MC-inducible mRNA with molecular size of 2.7 kb and, analogously, we observed a 1.9-kb, 3-MC-inducible mRNA upon hybridization of total rat liver RNA to the P-450d oligomer. These sizes are in good agreement with those predicted from the reported mRNA sequences (23, 24). In the adult rat, we observed P-450d mRNAs in total RNA isolated from control animals but little or no P-450c mRNA. Following 3-MC treatment, the P-450d mRNA level accumulated to an amount which surpassed the induced P-450c level. Neither mRNA species was induced following PB treatment. These findings in the adult rat liver corroborate those obtained

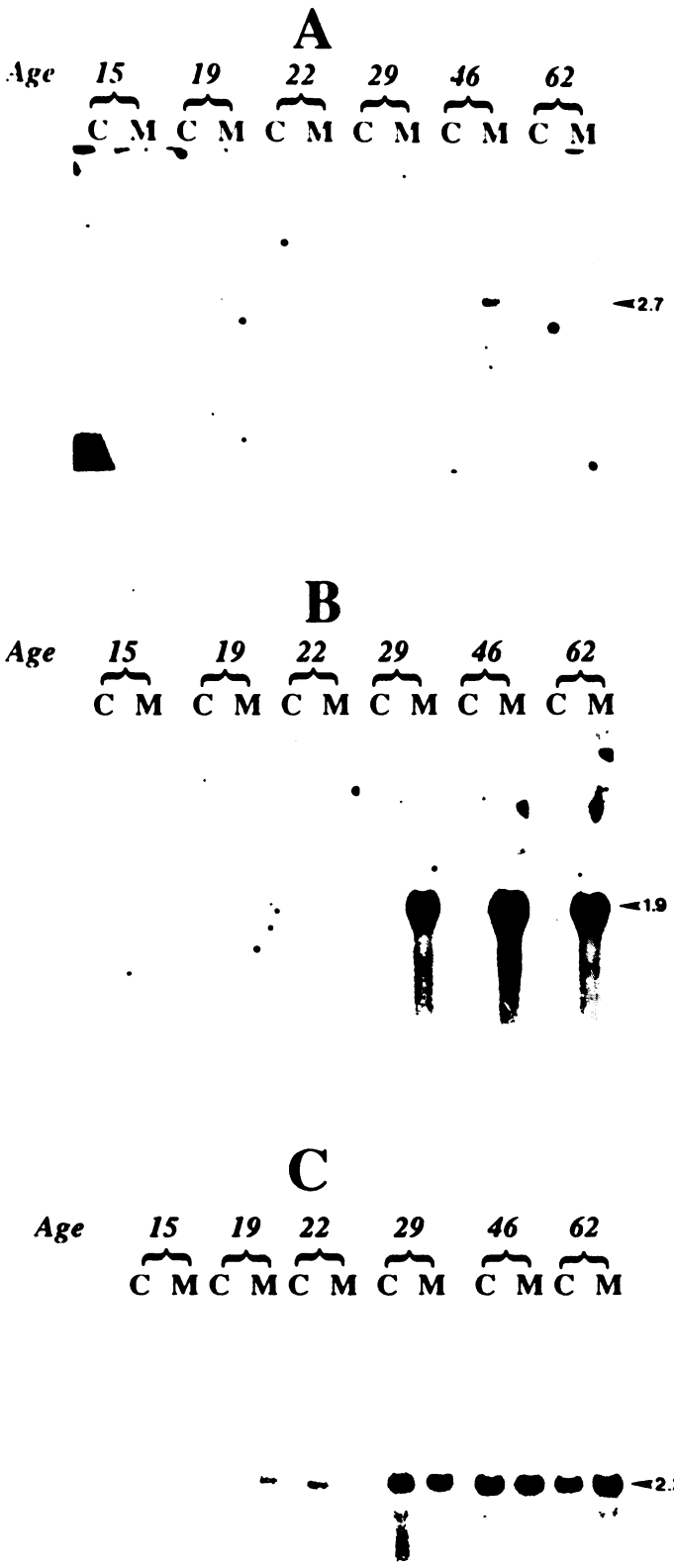

Fig. 6. Northern blot analysis of rat hepatic P-450c, P-450d, and albumin mRNA during development. Twenty μg of total hepatic RNA from control (*C*) and 3-MC-treated (*M*) rats were probed with [^{32}P]-P450c (A), [^{32}P]-P450d (B), and [^{32}P]-pRSA57 (C) probes. Age is given in days after conception. Molecular size is indicated in kilobases.

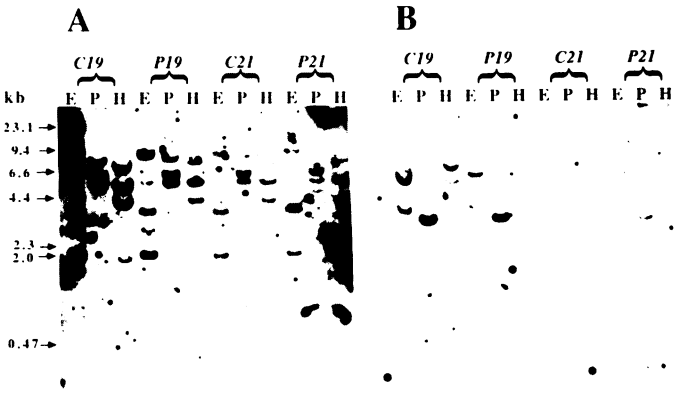

Fig. 7. Genomic blot analysis of P-450b and P-450e genes during development. Fifteen μg of DNA from fetal rats were restricted with either *Eco R1* (*E*), *Pst* 1 (*P*), or *Hind* 111 (*H*) and subjected to electrophoresis. The DNA was transferred to GeneScreen Plus and hybridized to [^{32}P]-P-450b (A) and [^{32}P]-P-450e (B) as described in Materials and Methods. Molecular sizes of concurrently run *Hind* 111 fragments are given in kilobases.

by Kawajiri et al. (29) using 3′-specific P-450c and P-450d cDNA probes.

We further utilized these oligomeric probes to study P-450c and P-450d mRNA levels at various stages in rat development. The results indicate that, in contrast to P-450b and P-450e mRNAs, both P-450c and P-450d mRNAs are detectable after inducer treatment only postnatally. These findings were repeated with identical results using two additional oligomer probes made complementary to different regions of the P-450c and P-450d mRNA, and with clone 46, a cDNA clone specific for $P_1$450 (data not shown). Similarly, Ikeda *et al.* (6) have shown that the highly homologous $P_3$450 mRNA is induced by MC only postnatally in B6 mice. In contrast, these researchers found that mouse hepatic $P_1$450 mRNA accumulates in response to 3-MC treatment by day 15 of gestation. It appears, therefore, that there is a species difference in the onset of 3-MC inducibility of P-450c- and P-450d-like genes in the mouse and rat. This variation in RNA accumulation correlates well with the species difference in the developmental appearance of the hepatic *Ah* receptor (30), suggesting that the delayed appearance of inducible P-450c mRNA can be attributed to the delayed expression of the regulatory gene product. In mice, the *Ah* receptor is detectable prior to birth, whereas in the rat only negligible levels can be detected prenatally (30).

However, previous studies of 3-MC-inducible enzymatic activities and protein levels in the rat generally suggest that, as in the mouse, a protein homologous to P-450c is inducible in fetal liver (9, 31). The fact that we do not observe P-450c mRNA prenatally in 3-MC-treated rats is probably not due to a lack of sensitivity of the oligonucleotide probe since, utilizing our labeling and hybridization conditions, as little as 0.01 fmol of specific mRNA is detectable. Assuming reasonable translational efficiencies and mRNA half-lives, this value compares well with reported sensitivity limits of Western blot experiments (0.50–0.1 pmol of protein). Our results might reflect an extremely short half-life of this mRNA species in fetal rat liver. Alternatively, these contradictory results might reflect the inherent problems associated with measuring specific P-450 isoenzymes with either enzymatic or immunological assays. Polyclonal antibodies to cytochrome P-450s commonly cross-react with homologous isozymes, and enzymatic assays are rarely selective for individual enzymes due to overlapping substrate specificities (32). In contrast, oligomer probing of mRNA has been shown to be a highly discriminating technique for examining individual P-450 isozyme levels (7). Consistent with our results is the finding by Lum *et al.* (33) that 3-MC failed to induce hepatic ethoxyresorufrin *O*-deethylase activity (catalyzed selectively by P-450c) prior to 1 week postpartum in the rat (33). Further studies are required to more fully understand the genetic regulation of 3-MC- and PB-inducible P-450 isoenzymes in the rat.

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