AN ABSTRACT OF THE THESIS OF

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The mammalian FMO represents a multigene family which oxygenates a large number of xenobiotics. No physiological role has been determined for FMO, although synthesis of disulfide bonds and detoxification of dietary chemicals have been suggested. Five FMO gene subfamilies, each containing a single gene, have been identified. In this study, we determined the patterns of fetal and neonatal development of FMO1 and FMO2 in rabbit liver and lung. The expression of two major isoforms, FMO1 and FMO2, in fetal and neonatal animals were characterized at the steady state levels of mRNA. Northern and slot blot analyses were performed with cDNA probes for each isoform to provide a qualitative and quantitative profile. In order to relate developmental changes in FMO to the

metabolism of xenobiotics for which lung is a target organ, the developmental expression of lung FMO (FMO2) mRNA is compared to rabbit CYP2B4 and CYP4B1 which are the major constitutive P450s in lung. The results show that the expression of FMO1 and FMO2 is tissue-dependent, although the mechanisms controlling the mRNA expression, such as rate of transcription, processing of primary RNA, efficiencies of nucleocytoplasmic transport and stability of RNA in the cytoplasm, are still unknown. The results indicate that the early development- and tissue-specific expression patterns of mRNA for FMO1 and FMO2 might play a significant role in the target organ toxicity of xenobiotics in the rabbit fetus and neonate.

Developmental Regulation of Flavin-Containing Monooxygenase (FMO) Form 1 and Form 2 mRNA in Fetal and Neonatal Rabbit

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Mei-Fei Yueh, Author

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INTRODUCTION

The mammalian flavin-containing monooxygenase (FMO) (EC 1.14.13.8) represents a multigene family which oxygenates a large number of heteroatom-containing xenobiotics, pesticides, and drugs. Unlike all other known oxidases and monooxygenases, the FMO forms a stable NADPH- and oxygen-dependent 4a-hydroperoxyflavin intermediate in the absence of an oxygenatable substrate and any soft nucleophile accessible to this intermediate will be oxidized (Ziegler, 1991; Ball and Bruice, 1980). This unique feature is responsible for the extraordinary range of substrate specificity of these flavoenzymes. Most compounds are oxidized to less toxic derivatives, however, with some substrates, highly reactive, toxic intermediates may be formed. In addition to its function in xenobiotic metabolism, no physiological role has been determined for the FMOs, although synthesis of disulfide bonds (Ziegler and Poulsen, 1977) and detoxification of dietary chemicals have been suggested as possibilities (Ayesh and Smith, 1990).

Characterization at the molecular level of the mammalian FMOs shows that a total of five FMO gene subfamilies, each containing a single gene, have been identified. A gene subfamily is defined by sequence identities between 52 and 57%, and orthologous proteins are defined as identities of greater than 80%.

Levels of FMO expression appear to be regulated developmently by age, sex and pregnancy in rabbit (Williams, 1991), and perhaps in humans (Sadeque et al., 1992). Hormonal, as well as tissue-specific factors, may play a key role in the regulation of FMO expression (Dannan et al., 1986; Lemoine et al., 1991). The distribution of FMO individual isoforms can be assessed immunochemically at the level of protein, catalytic activity toward specific substrates, and unique stereoselectivity toward selected chiral substrates relative to other oxygenases and among individual FMOs.

In this study, I characterized the expression of two major isoforms, FMO1 and FMO2, in fetal and neonatal rabbit tissues at the steady state levels of mRNA. We have been able to characterize the mRNA levels in the developmental regulation of FMO1 and FMO2 with northern and slot blot analyses. The tissue-specific distribution of mRNA for FMO1 and FMO2 indicates that some unknown factors control the enzyme mRNA expression and/or stability.

Catalytic Mechanism

The FMO was originally isolated from hog liver and characterized on the basis of its ability to catalyze the N-oxygenation of N,N-dimethylaniline (Ziegler and Mitchell, 1972). Prior to 1979, this flavoprotein was referred to as an N-oxidase or mixed-function amine oxidase. However, this name was too narrow since it soon became evident that many FMO substrates with low K_ms lacked

nitrogen.

The catalytic cycle, shown in Figure 1, illustrates the major steps characteristic of FMO. The studies on mechanism, based on detailed kinetic and spectral studies with FMO purified from hog liver microsomes, (Poulsen and Ziegler, 1979; Beaty and Ballou, 1981a; 1981b) demonstrated that FMO probably exists *in vivo* in the very reactive 4a-hydroperoxyflavin form and any soft nucleophile that can gain access to this potent monooxygenating agent will be oxidized (Ball and Bruice, 1980). Unlike all other monooxygenases, the oxygenatable substrate is not required for dioxygen reduction by NADPH (Ziegler, 1991). The product (SO), formed by oxygen transfer from the hydroperoxyflavin to the nucleophile, is released immediately. Steps 2-5 simply regenerate the enzyme-bound peroxyflavin intermediate from NADPH and oxygen and, as stated above, the substrate is not required for any of these steps.

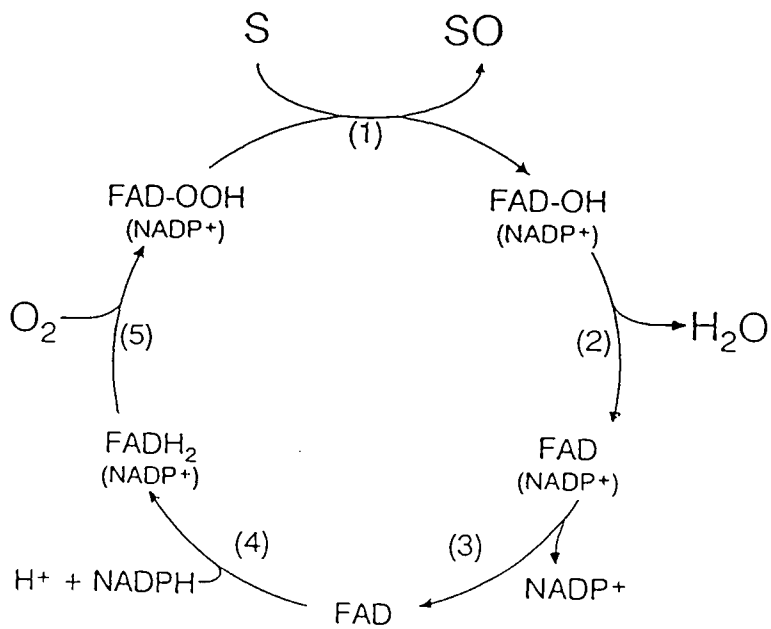
Because the energy for catalysis is present in the enzyme before contact with the xenobiotic, the fit of substrate is not as stringent as with most enzymes. A single point contact between the xenobiotic and the terminal oxygen of the hydroperoxyflavin is all that is required for product formation. This feature, unique to FMOs, is responsible for the enzyme's ability to metabolize a wide variety of xenobiotics. These include tertiary and secondary alkyl- and arylamines, many hydrazines, thiocarbamides, thioamides, sulfides, disulfides, thiols, and other soft nucleophiles (Poulsen, 1991; Ziegler, 1988; Prough et al., 1981).

Despite a mechanism designed for loose specificity, these enzymes possess some ability for discriminating between essential

and xenobiotic nucleophiles. The number and position of ionic groups appears to be the principal determinate used to discriminate among different nucleophiles (Ziegler, 1990). Almost without exception, essential metabolites bearing nucleophilic sulfur, nitrogen, or selenium atoms contain one or more charged groups that prevent entry into the active site.

Fig. 1 Major steps in the catalytic cycle of FMO
(Ziegler, 1991)

Oxygenated product (SO) is formed by nucleophilic attack of oxygenatable substrate (S) on the terminal oxygen of the enzyme-bound hydroperoxyflavin followed by heterolytic cleavage of the peroxide (step 1)



Multiple Forms

The possibility that multiple forms of FMO might exist was first suggested by Devereux et al. (1977), who observed that Hg^{+2} and Mg^{+2} increased the rate of dimethylaniline N-oxidation catalyzed by FMO partially purified from rabbit lung, but decreased the rate mediated by a similar preparation from rabbit liver. Analysis of the oxidation of dimethylaniline, imipramine, and chlopromazine in hepatic and pulmonary microsomal preparations from rabbit and rat reinforced the hypothesis that there were multiple isoforms of FMO expressed in different tissues of rabbit (Ohmiya and Mehendale, 1982; 1983; 1984). Purification and characterization of a flavin-containing monooxygenase from rabbit lung provided conclusive evidence that distinctly different FMOs can be isolated from lung and liver (Willlams et al., 1984; Tynes et al., 1985; Tynes and Philpot, 1987). In addition to immunological differences, the major hepatic and pulmonary forms differ in thermal stability, optimal pH, and sensitivity to anionic detergents (Williams et al., 1985; Tynes et al., 1985). The structural and functional differences between the liver and lung FMOs led to a hypothesis for the existence of two distinct FMO genes. Confirmation of this hypothesis by direct sequencing of the FMOs was prevented by blocked N-termini, and isolation of peptides from proteolytic digestion proved difficult.

The application of molecular biology techniques to this enzyme family has contributed to knowledge concerning the expression, structure and function of the FMO isoforms. Polyclonal antibodies to the pig liver FMO were used to screen a pig liver cDNA library, and a

protein of 532 amino acids was found (Gasser et al., 1990). This cDNA was then used to isolated a clone from a rabbit liver cDNA library (Lawton et al., 1990). Based on a similar approach, a cDNA encoding the rabbit lung FMO was also isolated and sequenced (Lawton et al., 1990). The possibility that more than one form of FMO might exist in rabbit liver was supported by Ozols who reported the sequences of two FMOs (about 55% identity) from rabbit liver (Ozols, 1989; 1990; 1991). The difference in the kinetic parameters of methimazole metabolism in the microsomal preparations from rabbit liver and lung and FMO1 and FMO2 cDNA expressed in COS-1 cells also indicated that additional FMO isoforms exist (Lawton et al., 1991). The search for cDNAs encoding additional isoforms of FMO was initiated by low stringency screening techniques. To date, 11 full length sequences from cDNAs and four from purified proteins have been reported in different species (Hines et al., 1994; Lawton et al., 1994). As shown in Table 1, there are at least five gene subfamilies for FMO (FMO1 through 5) which are expressed in a species- and tissue-specific manner. The primary structures of the five FMO isoforms from rabbit are from 52-57% identical. Orthologs of the rabbit isoforms, present in other mammals, including human, guinea pig, and rat, are from 86-88% identical in sequence to the rabbit forms (Hines et al., 1994; Lawton et al., 1994). Results of analyses of genomic DNA by Southern blotting indicate that each gene subfamily contains a single gene. The consensus sequence around the flavin (residues 9 to 14) and NADPH (residues 191 to 196) binding sites are identical in all forms of mammalian FMO. The

region from 325 to 400 is also highly conserved but there is little sequence identity near the C-terminus (Ziegler, 1993).

TABLE 1 *

Reported Sequences of Flavin-Containing Monooxygenases

Proposed name	Trivial name	Species	Source	Reference
FMO1	1A1	Rabbit	cDNA	(Lawton et al., 1990)
	Form1	Rabbit	Protein	(Ozols, 1990)
	Liver	Porcine	cDNA	(Gasser et al., 1990)
	FMO1	Human	cDNA	(Dolphin et al., 1991)
	RFMO1	Rat	cDNA	(Itoh et al., 1993)
FMO2	1B1	Rabbit	cDNA	(Lawton et al., 1990)
	Lung	Rabbit	Protein	(Guan et al., 1991)
	1B1	Guinea pig	cDNA	(Nikbakht et al., 1992)
FMO3	1D1	Rabbit	cDNA	(Burnett et al., 1992)
	Form2	Rabbit	Protein	(Ozols, 1991)
	FMOII	Human	cDNA	(Lomri et al., 1992)
FMO4	1E1	Rabbit	cDNA	(Burnett et al., 1992)
	FMO2	Human	cDNA	(Dolphin et al., 1992)
FMO5	1C1	Rabbit	cDNA	(Atta-Asafo-Adjei et al, 1993)
	Form3	Rabbit	Protein	(Ozols, 1992)

* Lawton et al., 1994

Substrate Specificity

The potential for a specific compound to be a substrate for a particular FMO can usually be evaluated based on two criteria. Firstly, only xenobiotics with an electron-rich center (soft

nucleophiles) can serve as substrates; secondly, it is evident that differences in access to the active site are largely responsible for differences in substrate specificity of various FMO isoforms.

Although the mechanism for oxidation of nucleophiles appears the same for all isoforms, differences in substrate specificities have been documented (Ziegler, 1988; 1991). The distinct substrate specificities of FMO were first detected in rabbit liver and lung microsomal preparations (Ohmiya and Mehendale, 1982; 1983; 1984). Some striking differences in the N-oxidation of amines with the purified flavoproteins also have been described (Williams et al., 1984, Tynes et al., 1986, Poulsen et al., 1986). For instance, FMO1 will not catalyze the oxidation of primary alkylamines, but FMO2 readily catalyzes the oxidation of this type of amine (Tynes et al., 1986). Also, FMO2 does not catalyze the oxidation of many tertiary amines which are good substrate for FMO1. Structure-activity studies with both organic nitrogen and sulfur nucleophiles demonstrated that access to the 4a-hydroperoxyflavin is quite different in these two FMO isoforms (i.e. FMO1, FMO2) (Nagata et al., 1990). The overall size of substrate and differences in the dimensions of the substrate channels of different FMO isoforms appear responsible for many of the distinct substrate specificities of these enzymes.

In the past, the activity of FMO was measured with whole homogenates and subcellular fractions by following the N-oxidation of dimethylaniline using a colorimetric method (Ziegler and Pettit, 1964). Subsequently, alternative methods for estimating the enzyme activity have also been described (Cashman, 1987; Cashman and

Hanzlik, 1981; McManus et al., 1983). Recently, isoform-selective probes for FMO catalytic activity in microsomal preparations have been developed (Guo and Ziegler, 1991; Guo et al., 1992; Cashman et al., 1992; Rettie et al., 1990). The procedure developed for measuring the oxidation of thiocarbamides to their sulfenic acids (Guo and Ziegler, 1991) is relatively simple and sensitive. The oxidation of five thiocarbamides, that varied in surface area, catalyzed by microsomes from different tissues and species was examined. Without exception, the specific activities decreased with increasing substrate size with all preparations tested (Guo et al., 1992). The pattern was also species- and tissue-specific. In addition, the tissue and species distribution of isoforms, determined by activity toward thiocarbamides, offers an explanation for the differences in the role of FMO in the N-oxidation of alkaloids by microsomes from different tissues and species. For instance, the overall size of an alkaloid as large as senecione would be readily catalyzed only by microsomes from pig and guinea pig liver, therefore, the contribution of FMO to the N-oxidation of this alkaloid is high in guinea pig liver (Miranda et al., 1991) but negligible in rat liver (Williams et al., 1989).

The contribution of FMOs to the metabolism of a given compound can also be assessed by its unique stereoselectivity relative to other oxygenases. For example, cytochromes P450 oxidize (S)-nicotine to a mixture of *cis*- and *trans*- N-1'-oxides. In contrast, (S)-nicotine oxidized by human FMO3 exclusively forms the *trans*-N-1'-oxide (Park et al., 1993). Among the individual members of the FMO gene family, the stereoselectivity of N- and S-oxygenation by

fetal human liver FMO1 (the major form in human fetal liver) (Sadeque et al., 1992) is quite distinct from that of adult human liver FMO3 (the major form in adult human liver) (Cashman et al., 1992; 1993a; Lomri et al., 1993a; 1993b). S-Monooxygenation enantioselectivity of cimetidine and the diasteroselectivity of tertiary amine N-1'-oxygenation of (S)-nicotine have been characterized (Cashman et al., 1992; 1993b) and the stereoselectivity of human urinary cimetidine S-oxide or (S)-nicotine N-1'-oxide metabolite formation can be used as a stereoselective marker for the presence of FMO isoforms expressed in the liver of humans and laboratory animals.

Further, the availability of cDNAs and appropriate expression systems also provide convenient models for observations on the catalytic activity of individual isoforms of FMO. FMO isoforms have been cloned into a number of heterologous expression systems including COS-1 cells, yeast, and *Escherichia coli* (Lawton et al., 1991; Atta-Asafo-Adjei et al., 1993; Lomri et al., 1993a; Lawton and Philpot, 1993). The availability of membrane-associated cDNA-expressed forms of FMO also eliminates many of the difficulties associated with the use of purified forms of the enzyme isolated by conventional chromatographic procedures. Recently, according to the consistent results obtained both from *E. coli* expression systems and liver microsomal preparations, methyl *p*-tolyl sulfide can be used as a suitable selective probe for discriminating between FMO1- and FMO3-mediated catalysis in rabbit liver microsomal preparations (Rettie et al., 1994).

Tissue and Species Distribution

The available evidence suggests that FMO is widely distributed in tissues and the amount of enzyme present varies with species and sex, but the highest concentration is usually found in the liver, with somewhat lesser amounts in lung and kidney (Dannan and Guengerich, 1982). Immunochemical measurements indicate that FMO makes up about 1% of the homogenate protein and more than 3% of the microsomal protein in liver from female hogs (Dannan and Guengerich, 1982). Sex differences in FMO concentration have been documented in rats, mice, and rabbits (Zielger, 1980; Dannan and Guengerich, 1982). The activity in liver from mice is usually higher in females than males, but this is reversed in rats. Five-fold induction of FMO in the placenta of mice during pregnancy has been reported (Omitz and Kulkarni, 1982) and the concentration of FMO in rabbit lung increases two-five fold during pregnancy and becomes the major microsomal protein, comprising 10% or more of the total (Williams et al., 1985).

Role in Metabolism

A large number of drugs in human clinical use today contain a nucleophilic nitrogen or sulfur. Two major monooxygenase systems considered to be responsible for heteroatom-containing chemical and drug oxidative metabolism include cytochrome P-450 (Guengerich, 1987) and FMO (Ziegler, 1980). The catalytic mechanism of FMO suggests that these enzymes have evolved to catalyze oxidative

detoxification of structurally diverse soft nucleophiles so abundant in food derived from plants (Liener, 1980). Without exception, aqueous extracts of plants, commonly consumed by humans, contain substrates for mammalian hepatic FMO (Ziegler, 1990). While the nature of these compounds has not been positively identified, various organic sulfur compounds derived from glucosinolates and endogenous nitrogen-containing alkaloids (Lovenberg, 1974) are the most likely candidates. Although, the majority of these compounds are detoxicated by FMO, several examples of metabolic activation to potentially toxic intermediates also exist. Thiocarbamides, for example, appear to be activated predominately by FMO. FMO catalyzes sequential S-oxygenation of thioureas to sulfinic acids through intermediate sulfenic acids (Decker and Doerge, 1991) which are potent thiol oxidants and the production of thiol reactive metabolites has been demonstrated both *in vitro* (Poulsen et al., 1979; Krieter et al., 1984) and *in vivo* (Krieter et al., 1984). Highly purified hog liver FMO catalyzed conversion of thiocarbamides to chemically reactive species, that covalently bound to protein thiols, has also been observed (Decker et al., 1992b). Because the parent xenobiotic can be regenerated upon reduction, these xenobiotics establish a futile cycle that oxidizes GSH to GSSG at the expense of NADPH and molecular oxygen (Poulsen and Ziegler, 1979). This process can deplete tissues of glutathione and reducing equivalents resulting in oxidative stress (Decker 1992a). However, it is likely that organic compounds oxidized to sulfenic acids preferentially react with glutathione and the first product is always the corresponding glutathione-xenobiotic mixed disulfide, and the mixed disulfides can

be excreted into bile (Ziegler, 1990). The latter pathway may be a significant route for detoxification and elimination of small amounts of sulfur xenobiotics oxidized to sulfenic acids via FMO. Recent studies also suggested that oxidation of selenides, catalyzed primarily by FMO1, may be a route for metabolic activation rather than detoxification. Based on K_m, both 2-selenylbenzanilide and 2-(methylseleno)benzanilide are excellent substrates for FMO1 and the selenoxides are potent thiol oxidants (Ziegler et al., 1992; Chen and Ziegler, 1994). Therefore, the oxidation of selenides catalyzed by FMO could establish a futile cycle leading to the oxidation of cellular thiols similar to that initiated by thiocarbamides (Krieter et al., 1984).

In human studies, the majority of evidence suggests that FMO3 is the dominant form of FMO expressed in adult human liver and probably accounts for most of the FMO-dependent N- and S-oxygenation in adult liver (Lomri et al., 1993a; 1993b; Sadque et al; 1992). In addition, it is likely that FMO from human fetal liver is functionally much more similar to FMO1 from animals than FMO3 from adult human liver (Sadque, 1992). How the developmental and pharmacological action of human liver FMO1 and FMO3 relate to their physiological role is still largely unknown. The N-oxygenation of trialkylamines is a significant route for the oxidative metabolism of a large number of medicinal amines and naturally occurring alkaloids with basic side chains (Damani, 1988). The studies of trimethylamine (TMA) metabolism in both chicken (Pearson et al., 1981) and man (Al-Waiz et al., 1987) suggest that oxidation of TMA to TMA N-oxide is catalyzed exclusively by FMO. The polymorphism

of TMA (Cholerton and Smith, 1991; Ayesh and Smith, 1990) and nicotine N-oxygenation (Ayesh et al., 1988) in humans apparently results from a genetic defect in FMO. In general, the role of the various FMO isoforms in metabolism of endogenous compounds, which might suggest a physiological function for FMO, remains unclear. Until recently, the conversion of cysteamine to yield cystamine was thought to be the only physiological reaction catalyzed by FMOs (Ziegler, 1993). However, a recent study suggests that methionine is a substrate for cDNA expressed rabbit FMO1, FMO2, and FMO3, although the K_ms are quite high (Duescher et al., 1994). This result indicates that FMO-catalyzed metabolism of methionine to methionine sulfoxide might become quantitatively significant when toxic levels of methionine are present in the diet, or in patients with a defect in the methionine transsulfuration pathway.

Regulation of FMO Expression

Unlike the P-450 system, mammalian FMO is not inducible by phenobarbital, 3-methylcholanthrene or any other class of P-450 inducer. An early attempt (Masters and Ziegler, 1971) to induce FMO by treating rats with phenobarbital either produced no change or decreased FMO activity. However, at that time, it was not known that FMO only catalyzes the oxidation of soft nucleophiles and the possible induction of these enzymes by dietary xenobiotics was therefore overlooked. Recent studies on FMO induction suggest that FMO is probably induced by one or more organic nitrogen- or sulfur-containing xenobiotic soft nucleophiles so abundant in food derived

from plants (Liener, 1980; Rosenthal and Janzen 1979). It is likely that FMOs regulated by xenobiotic nucleophiles are already maximally induced in animals maintained on commercial rat chow and induction can be observed only after the animals are maintained on a diet relatively free from such xenobiotics (Kaderlik et al., 1991). Recently, the effects of diets relatively free from xenobiotics on the pharmacokinetics of ethylmethylsulfide and trimethylamine in rats were observed (Ziegler, 1993). The *in vivo* disposition of both xenobiotics were significantly altered in animals on a semisynthetic diet and the decreased clearance of both correlated with the changes in the concentration of liver FMO. However, it can not be ruled out that other enzymes could be involved.

As mentioned above, an inducer for FMO has not been identified so far. However, FMO does appear to be regulated developmentally in a number of species and tissues. The majority of evidence suggests that FMO isoforms are expressed in different tissues as a function of development or hormone manipulation. These factors include sex, age, oestrus cycle and pregnancy (Williams, 1991). A five-fold induction in FMO in the placenta of mice during pregnancy has been reported (Omitz and Kulkarni, 1982). Liver from guinea pigs supplied commercially vary seasonally over 30-fold with respect to FMO activity (Ziegler, personal communication). Previous work also indicated that FMO was induced during pregnancy (Devereux and Fouts, 1975) and the concentration of FMO in rabbit lung increased five-fold and became the major microsomal protein in lung tissue from pregnant rabbits (Williams et al., 1985). The FMO2 protein and mRNA expression varied in rabbit lung during pregnancy

with peak levels correlated to elevated progesterone and cortisol plasma concentrations. The effect of exogenous steroid administration on the induction FMO2 protein and catalytic activity in rabbit lung microsomes also supported an involvement of progesterone and glucocoticoids (Lee et al., 1993). In the female mouse, liver FMO may be under nutritional and diurnal control (Dixit and Roche, 1984) and correlation of hepatic activity with serum corticosterone levels in inbred strains of mice has been described (Padilla et al., 1980). Studies employing gonadectomy of young animals indicate a role for sex steroids in regulation of FMO (Duffel et al., 1981; Dannan et al., 1986). These studies indicate that the concentration of FMOs is under hormonal control, and the level of FMO protein and catalytic activity appear to be regulated during development, but the effects appear to be species- and tissue-dependent.

In this study, I expand the study of the developmental regulation of FMO. We followed the development of FMO1 and FMO2 in liver and lung of fetal and neonatal rabbits from gestation through three weeks post-partum and expression for FMO1 and FMO2 is characterized by determining mRNA levels. In order to relate developmental changes in FMO to the metabolism of xenobiotics for which lung is a target organ, the developmental expression of lung FMO (FMO2) mRNA is compared to CYP2B4 and CYP4B1 which are the major constitutive P450s in rabbit lung. A understanding of the developmental regulation of FMO isoforms in fetal and neonatal animals is critical in evaluating their potential role in drug metabolism and toxicity during this critical and vulnerable time. The

results suggest that the early development and tissue-specific expression patterns of FMO mRNA might have a significant role in the target organ toxicity of xenobiotics to which the fetus and neonate is exposed.

MATERIALS AND METHODS

Reagents

Total RNA isolation reagent (TRI REAGENT) and FORMAzol were obtained from Molecular Research Center, Inc., Cincinnati, OH. The digoxigenin (DIG)-DNA labeling and detection kit, oligonucleotide 3'-end labeling kit, blocking reagent, anti-DIG Fab fragment- alkaline phosphatase (anti-DIG:AP) and lumigen PPD were obtained from Boehringer Mannheim Corp., Indianapolis, IN. Restriction enzymes (BamHI, EcoRI, AvrII, NcoI) were obtained from New England Biolabs, Inc., Beverly, MA. Nylon membrane (Hybond N^{+}) and Hyper film MP were purchased from Amersham Corp., Arlington Heights, IL. NaCl, Na_2HPO_4, NaH_2PO_4, NaOH, Tris, agarose, formaldehyde, sodium acetate, EDTA, diethylpyrocarbonate (DEPC), methylene blue, bromophenol blue, glycerol and Tween 20 were from Sigma Chemical Co., St, Louis, MO. Sodium dodecyl sulfate (SDS), maleic acid, 3-(N-morpholino)-propane-sulfonic acid (MOPS) and formamide were from US Biochemical Corp., Cleveland, OH.

Animals and Tissues

Timed-pregnant New Zealand white rabbits were obtained from the Rabbit Research Center at Oregon State University, and housed individually at the Laboratory Animal Resource Center.

Three rabbits were euthanized by CO_2 asphyxiation on days 0, 12, 15, 18, 21, 25, 28, and 31 days of gestation and then also at 1, 8 and 21 days postpartum. Fetuses and kits were killed at the same time to follow the appearance and development of FMO and their tissues (liver and lung) were immediately frozen in liquid nitrogen and stored at -80° C until assayed.

Total RNA Isolation

Total RNA was extracted from rabbit fetal and neonatal tissues (liver and lung) by using TRI REAGENT which is composed of phenol and guanidine thiocyanate in a monophase solution (Chomczynski, 1993). Sample tissues were homogenized in TRI REAGENT. After addition of chloroform and centrifugation, the aqueous phase was removed and total RNA was precipitated by isopropanol, washed with ethanol and dissolved in the RNA storage solution, FORMAzol. The amount of RNA in each sample was quantified by spectrophotometric measurement at 260 and 280 nm. An OD of 1 corresponds to approximately 40 μg/ml for RNA.

Rabbit FMO1, FMO2, CYP2B4 and CYP4B1 cDNA Probes

The rabbit FMO1 and FMO2 cDNAs used in this study were prepared in Dr. Hines' Lab (Wayne State University, Detroit, MI). mRNA was isolated from liver and lung of female rabbits at 28 days of gestation to produce cDNA libraries. Oligonucleotide N-terminal sense and C-terminal antisense primers for the rabbit liver FMO1

and rabbit lung FMO2 isozymes (Lawton et al., 1990) were synthesized and used to amplify the FMO cDNA from a total cDNA library. The resulting FMO1 1641 bp fragment, spanning from 34 to 1674 was cloned into the SmaI site of pBluescriptIIKS$^+$ and identified as pRNH141. The FMO2 1644 bp fragment spanning from 67 to 1710 was cloned into SmaI site of pUC19 and identified as pRNH135. The cDNAs encoding rabbit CYP2B4 and CYP4B1 were obtained from Dr. Richard Philpot's Lab (NIEHS, Research Triangle Park, NC) and contain 2067 bp and 1760 bp, respectively.

Probe Labeling

The full length cDNA encoding rabbit FMO1 and FMO2 were digested with the restriction enzymes EcoRI/BamHI and AvrII/EcoRI, respectively. The fragments were labeled with DIG-11-dUTP using the random priming method. Based on the same approach, after the EcoRI digestion, the cDNA fragments for CYP2B4 and CYP4B1 were labeled with DIG-11-dUTP.

Northern Blot Analysis

Samples of total RNA (20 μg) from lung were separated by electrophoresis in a 1% agarose gel containing 0.5 M formaldehyde in MOPS buffer (20 mM MOPS, pH 7.0; 5 mM sodium acetate; 1 mM EDTA, pH 8.0) (Sambrook et al., 1989; Farrel, 1993) and transferred by a downward capillary method (Chomczynski, 1992) to a nylon membrane. The RNA was immobilized by UV-crosslinking (UV

crosslinker, XL-1000, Fisher Scientific., Pittsburgh, PA) and baked for 60 min at 68°C. Following the transfer, the membrane was prehybridized for a minimum of 1 hr at 68°C (16.3% SDS; 0.25 M Na_2HPO_4, pH 7.2; 1 mM EDTA; 5% blocking reagent), then hybridized overnight at 68°C with a random primer DIG-dUTP-labeled cDNA probe which encodes rabbit FMO2 (Engler-Blum et al., 1993). Hybridization was carried out in a Hybridization Incubator (Model 310, Robbins Scientific, Sunnyvale, CA). Hybridized membrane was washed at 68°C (Engler-Blum et al., 1993). After the wash, detection of DIG-labeled mRNA was accomplished by a chemiluminescence detection method (Engler-Blum et al., 1993). Detection was carried out by first washing the membrane in washing buffer (100 mM maleic acid, pH 8.0; 3 M NaCl; 0.3% Tween 20). After the membrane was incubated in blocking buffer (100 mM maleic acid, pH 8.0; 3 M NaCl; 0.3% Tween 20; 0.5% blocking reagent) for 60 min, anti-DIG:AP conjugates were diluted 1:15000 in blocking buffer and the membrane was incubated for 30 min and then washed with washing buffer (100 mM maleic acid, pH 8.0; 3 M NaCl; 0.3% Tween 20) three times for a total of at least 1 hr for removal of unbound antibody-conjugates. Lumigen PPD was utilized as the chemiluminescent substrate and membrane was exposed to a Hyper-film for various times (20 min to 8 hr), depending on the signal intensity

Slot Blot Analysis

Total RNA, purified from rabbit fetal and neonatal tissues (liver and lung), was applied to a slot blotter (Farrel, 1993), followed by

RNA fixation, prehybridization, hybridization, post hybridization wash, chemiluminescent detection and autoradiography, as described above. Samples were hybridized with full-length rabbit FMO1 cDNA (1662 bp), rabbit FMO2 cDNA EcoRI/AvrII fragment (1222 bp), full length CYP2B4 (2067 bp) or CYP4B1 (1760 bp) cDNAs.

Normalization of RNA

RNA slot blots were normalized by hybridization with a 16S rRNA oligonucleotide to control for variations in loading of the total RNA samples. This oligonucleotide probe (30 mer) was labeled with DIG-11-dUTP by a 3'-end labeling method and hybridized for 3 hr at 41° C. The process for hybridization and chemiluminescent detection was based on the same protocol as described above.

Autoradiogram Scanning

Quantification of the amount of hybridized RNA on the slot blot was based on a measurement of area and density and performed by a HP scanner interfaced into a computer and using NIH image 1.54 software.

RESULTS

Northern Analysis of Total RNA Isolated From Rabbit Lung with FMO2 cDNA Probe

Northern blot revealed multiple bands of FMO2 mRNA in lung (Fig. 1). These previously observed bands (Lawton et al., 1990)) might be indicative of alternative processing of the 5' and 3' flanking region of the transcript, the latter being consistent with the presence of multiple polyadenylation signals for FMO2 cDNA.

Expression of FMO1 mRNA in Liver From Fetal and Neonatal Rabbit

Figure 3 shows a slot blot membrane of total RNA from rabbit fetal and neonatal liver probed with cDNA encoding rabbit FMO1. Quantification of mRNA levels (Fig. 3) revealed that FMO1 increased gradually during gestation. Following parturition, FMO1 levels were low in one day old neonates then rose dramatically, although a high degree of individual variation was observed (Fig. 5). We also probed the adult lung RNA with the FMO 1 cDNA probe and zero to trace amounts of FMO1 mRNA were detected in lung.

Expression of FMO2 mRNA in Lung From Fetal and Neonatal Rabbit

Figure 6 shows the slot blot analysis of total RNA from rabbit fetal and neonatal lung with cDNA encoding rabbit FMO2. At the 25th day of gestation, fetuses from rabbit A and B appeared to express higher FMO2 mRNA levels than that of adult male, but no detectable FMO2 could be seen in fetuses from rabbit C (Fig. 7). Interestingly, no FMO2 mRNA was detected at the 28th day of gestation in all three rabbits examined. These results were not due to mRNA degradation as electrophoretic analysis demonstrated no difference between these samples and those exhibiting high FMO2 mRNA levels. At the 31th day of gestation, the levels of FMO2 mRNA were 3-fold greater than adult male levels in all 3 rabbits.

Following parturition (Fig. 8), levels of FMO2 mRNA were as high or higher than adult male, although, there was a high degree of individual variation at the 21st day postpartum. Undetectable to trace amounts of FMO2 mRNA were seen in control adult and fetal liver RNA samples.

Expression of CYP2B4 and CYP4B1 mRNA in Lung From Fetal and Neonatal Rabbit

Figures 9 and 12 show slot blot analysis of total RNA from rabbit fetal and neonatal lung probed with cDNA encoding rabbit CYP2B4 or CYP4B1, respectively. Quantification of the results from slot blot analysis revealed that mRNA for CYP2B4 and CYP4B1 were significant at the first time point examined, the 25th day of gestation,

and fetuses from rabbit A and B appeared to express higher mRNA level in both CYP2B4 and CYP4B1 than that of adult male. As mentioned above, FMO2 mRNA levels in fetuses from 25A and 25B are also higher than adult male levels and similar distribution patterns of CYP2B4 and CYP4B1 also occurred on the 28th and 31th day of gestation, compared to FMO2 levels. Undetectable CYP2B4 and undetectable to trace amounts of CYP4B1 were observed at the 28th day of gestation the, CYP2B4 and CYP4B1 rose to 74% and 120% of adult levels on the 31st day of gestation, respectively. Following parturition, interestingly, CYB2B4 mRNA was undetectable on the 1st day postpartum in all three neonates, rose again to 30% of adult levels at the 8th day postpartum, although a high degree of individual variation was observed. CYP2B4 mRNA levels were 62% of adult levels at the last point examined of 21 days. CYP4B1 levels increased steadily through the 1st, 8th and 21st day postpartum and were 60%, 71% and 78.5% of adult levels, respectively (Figs. 10, 11 13, 14).

Fig. 2 Northern blot analysis of total RNA from rabbit lung.

Samples of total RNA from rabbit lung (10 μg each) were subjected to electrophoresis in agarose, transferred to nylon membrane, and probed with rabbit lung cDNA labeled with DIG.

RNA marker

Fig. 3 Slot blot analysis of total RNA from rabbit fetal and neonatal liver, and control sample lung with cDNA encoding rabbit FMO1.

Samples of total RNA (10 μg) were applied to a slot blotter and membranes hybridized to FMO1 cDNAs labeled with DIG by the random priming method.
Top figures in (A) (B) (C) and (D) represent FMO1 cDNA hybridization.
(A) Lanes 1 through 12 (25A, 25B, 25C, 28A, 28B, 28C, 31A, 31B, 31C, 1ppA, 1ppB, 1ppC).
THe number (25, 28, or 31) indicates the day of gestation and the letter denotes that these were pooled fetuses from a single maternal rabbit. The number followed by pp indicates the days post-partum. The letters denote distinct litters.
(B) Lanes 1 through 3 ($8ppB_1$, B_2, B_3). (B) Lanes 4 through 6 (10 μg of lung RNA from 1ppA, adult male and adult female). (B) Lanes 7 through 11 (adult male liver RNA 1, 2.5, 5, 10, and 15 μg).
(C) Lanes 1 through 12 ($8ppC_1$,C_2, C_3, C_4, $21ppA_1$, A_2, A_3, A_4, A_5, A_6, A_7, B_1).
(D) Lanes 1 through 3 ($21ppB_2$, B_3, B_4) (D) Lanes 4 and 5 (10 μg of lung RNA from 1ppA and adult male). (D) Lanes 7 through 11 (adult male liver RNA 1, 2.5, 5, 10, and 15 μg)
The number followed by pp indicates the days post-partum. The letters denote distinct litters and the numbered subscripts indicate individual neonates.
Bottom figures show 16S rRNA hybridization for each.

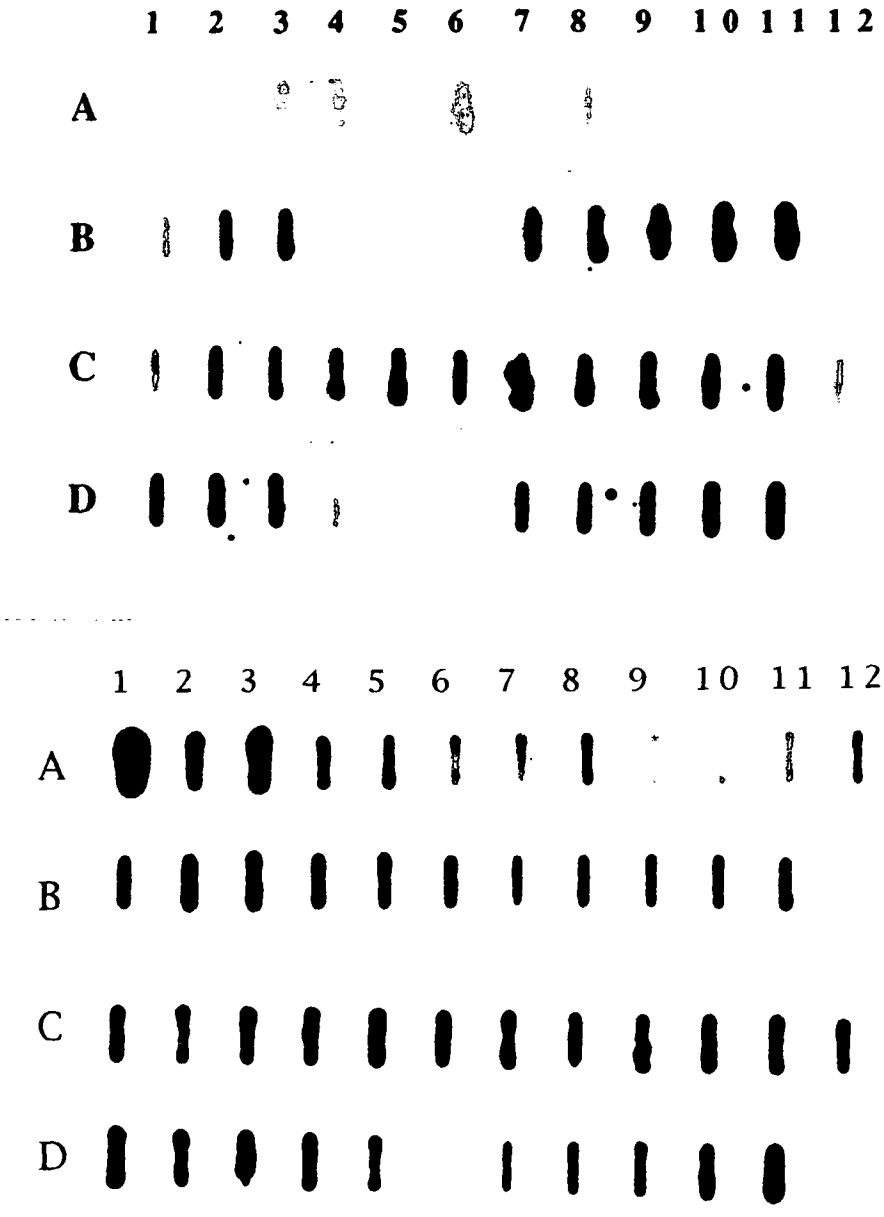
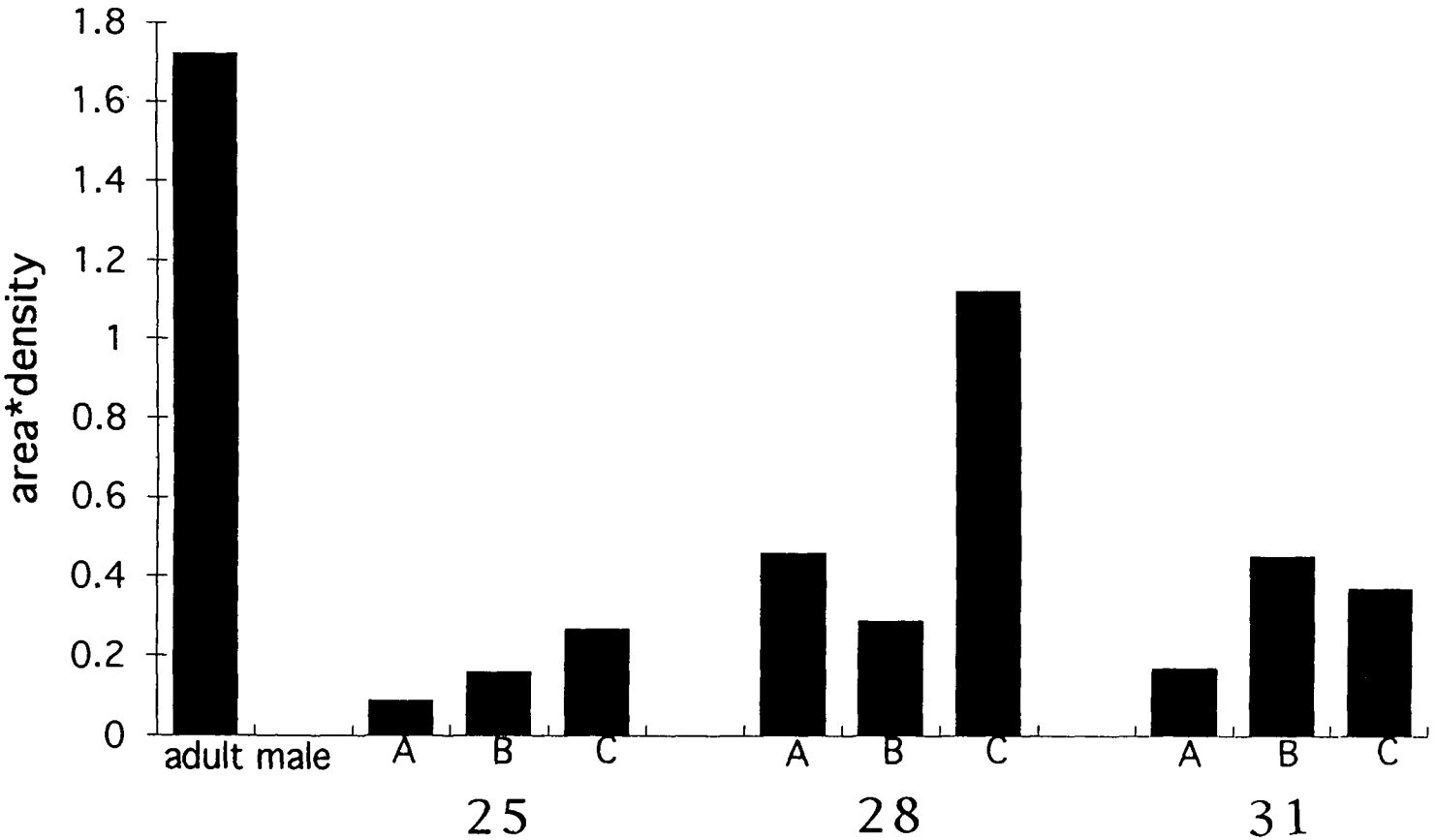


Figure 3

Fig. 4 Quantitation of FMO1 mRNA in liver from fetal rabbit.

Samples of total RNA (10 μg) were applied to a slot blotter and the membrane hybridized to rabbit FMO1 cDNA (1622 bp) labeled with DIG by the random priming method. The data are expressed as area* density in arbitrary units after scanning densitometry as described in the materials and methods.
The number on the x-axis indicates the day of gestation and the letter denotes that these were pooled fetuses from a single maternal rabbit.


area*density
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1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
adult male
A
B
C
A
B
C
A
B
C
25
28
31

Figure 4

Fig. 5 Quantitation of FMO1 mRNA in liver from neonatal rabbit.

Samples of total RNA (10 μg) were applied to a slot blotter and the membrane hybridized to rabbit FMO1 cDNA (1622 bp) labeled with DIG by the random priming method. The data are expressed as area* density in arbitrary units after scanning densitometry as described in the materials and methods.
The number on the x-axis followed by pp indicates the days post-partum. The letters denote distinct litters and the numbered subscripts indicate individual neonates.

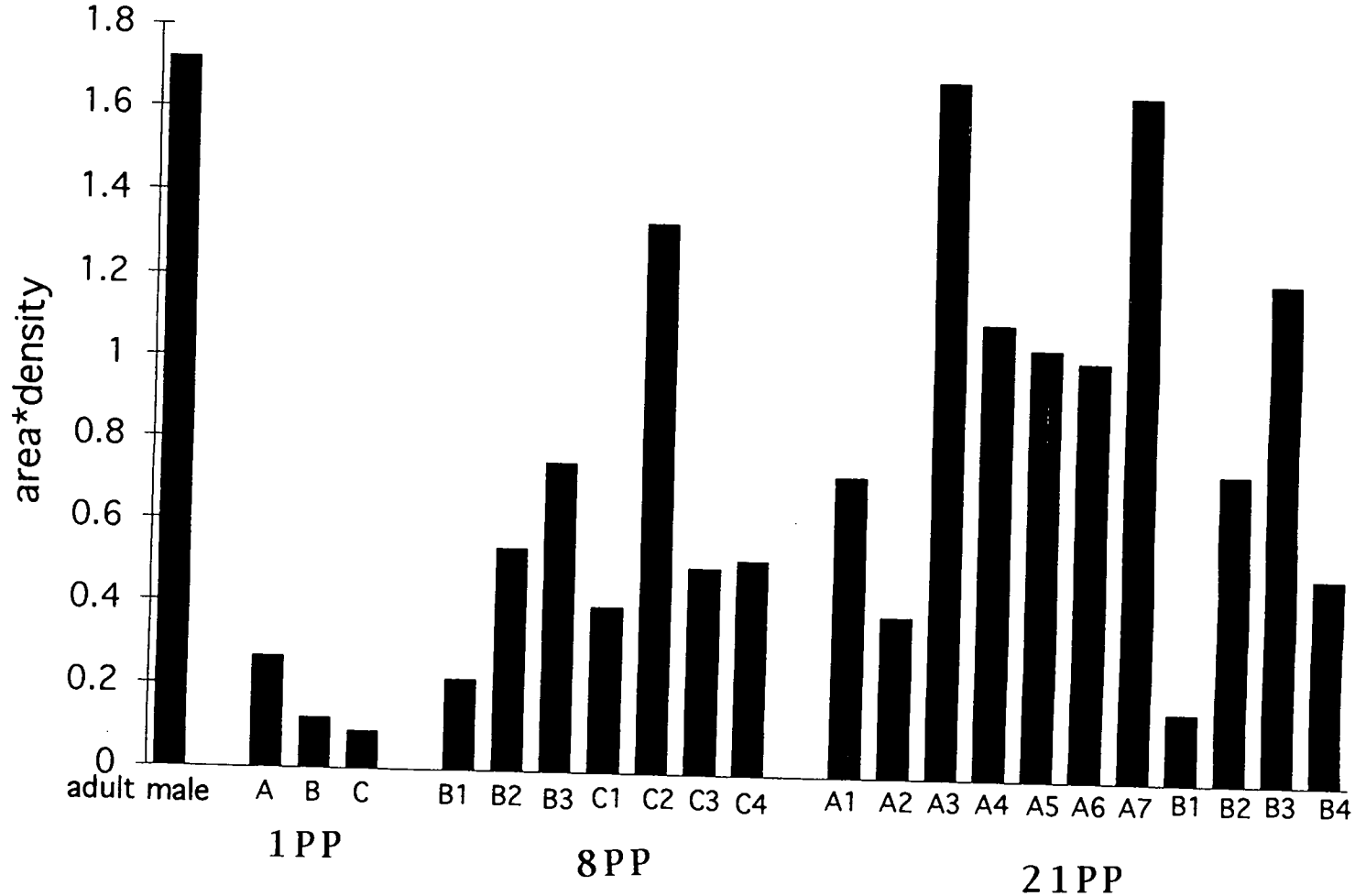
area*density
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
adult male
A B C
B1 B2 B3 C1 C2 C3 C4
A1 A2 A3 A4 A5 A6 A7 B1 B2 B3 B4
1PP
8PP
21PP

Figure 5

Fig. 6 Slot blot analysis of total RNA from rabbit fetal and neonatal lung, and control sample liver with cDNA encoding rabbit FMO2.

Samples of total RNA (4 μg) were applied to a slot blotter and membranes hybridized to FMO2 cDNAs labeled with DIG by the random priming method.
Top figures in (A) (B) (C) and (D) represent FMO2 cDNA hybridization.
(A) Lanes 1 through 12 (25A, 25B, 25C, 28A, 28B, 28C, 31A, 31B, 31C, 1ppA, 1ppB, 1ppC).
THe number (25, 28, or 31) indicates the day of gestation and the letter denotes that these were pooled fetuses from a single maternal rabbit. The number followed by pp indicates the days post-partum. The letters denote distinct litters.
(B) Lanes 1 through 3 (8ppB_1, B_2, B_3). (B) Lanes 4 through 5 (5 μg of liver RNA from 25A fetuses, adult male). (B) Lanes 6 through 11 (adult male lung RNA 0.2, 1, 2.5, 5, 10, and 15 μg).
(C) Lanes 1 through 12 (8pp C_2, C_3, C_4, 21pp A_1, A_2, A_3, A_4, A_5, A_6, A_7, B_1, B_2).
(D) Lane 1 (21pp B_3) (D) Lanes 4 and 5 (5 μg of liver RNA from 25A fetuses and adult male). (D) Lanes 6 through 11 (adult male lung RNA 0.2, 1, 2.5, 5, 10, and 15 μg)
The number followed by pp indicates the days post-partum. The letters denote distinct litters and the numbered subscripts indicate individual neonates.
Bottom figures show 16S rRNA hybridization for each.

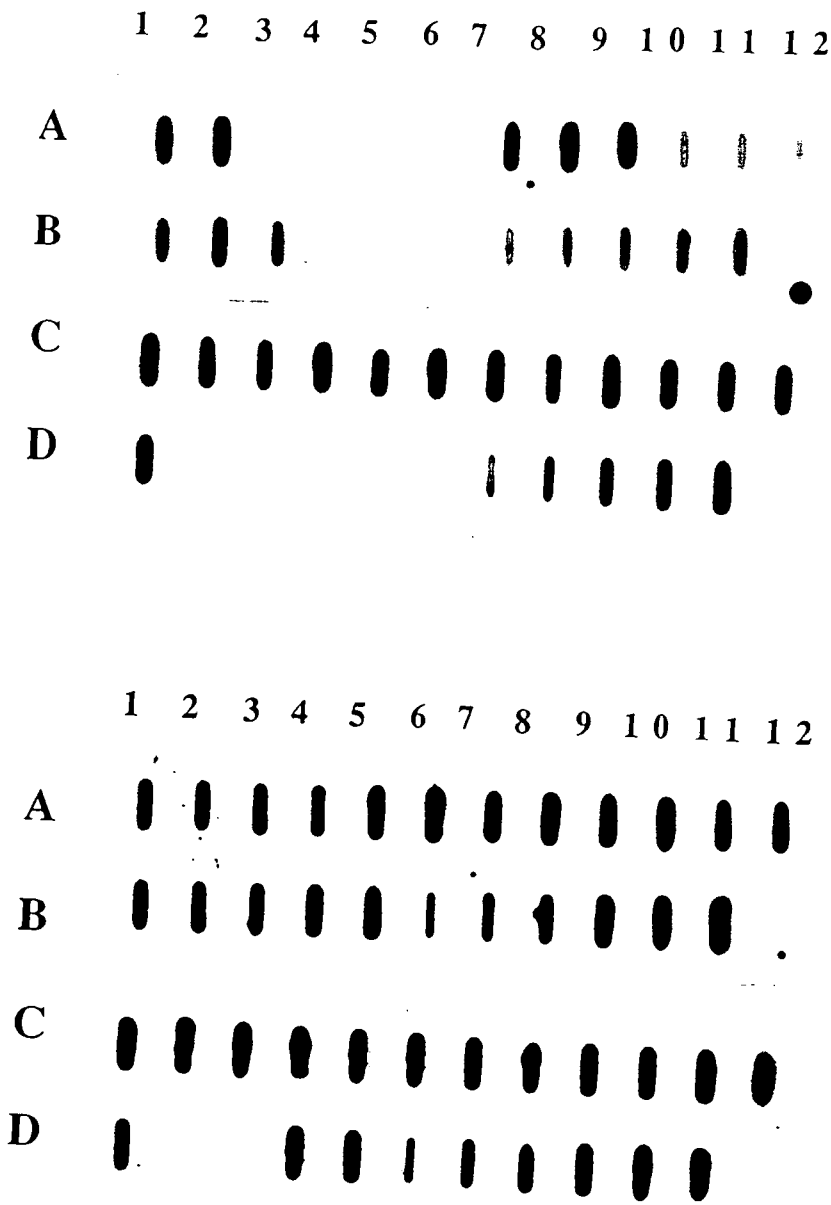

Figure 6

Fig. 7 Quantitation of FMO2 mRNA in lung from fetal rabbit.

Samples of total RNA (4 μg) were applied to a slot blotter and the membrane hybridized to rabbit FMO2 cDNA (1142 bp) labeled with DIG by the random priming method. The data are expressed as area* density in arbitrary units after scanning densitometry as described in the materials and methods.
The number on the x-axis indicates the day of gestation and the letter denotes that these were pooled fetuses from a single maternal rabbit.
25C, 28A, 28B and 28C had no detectable FMO2 mRNA.

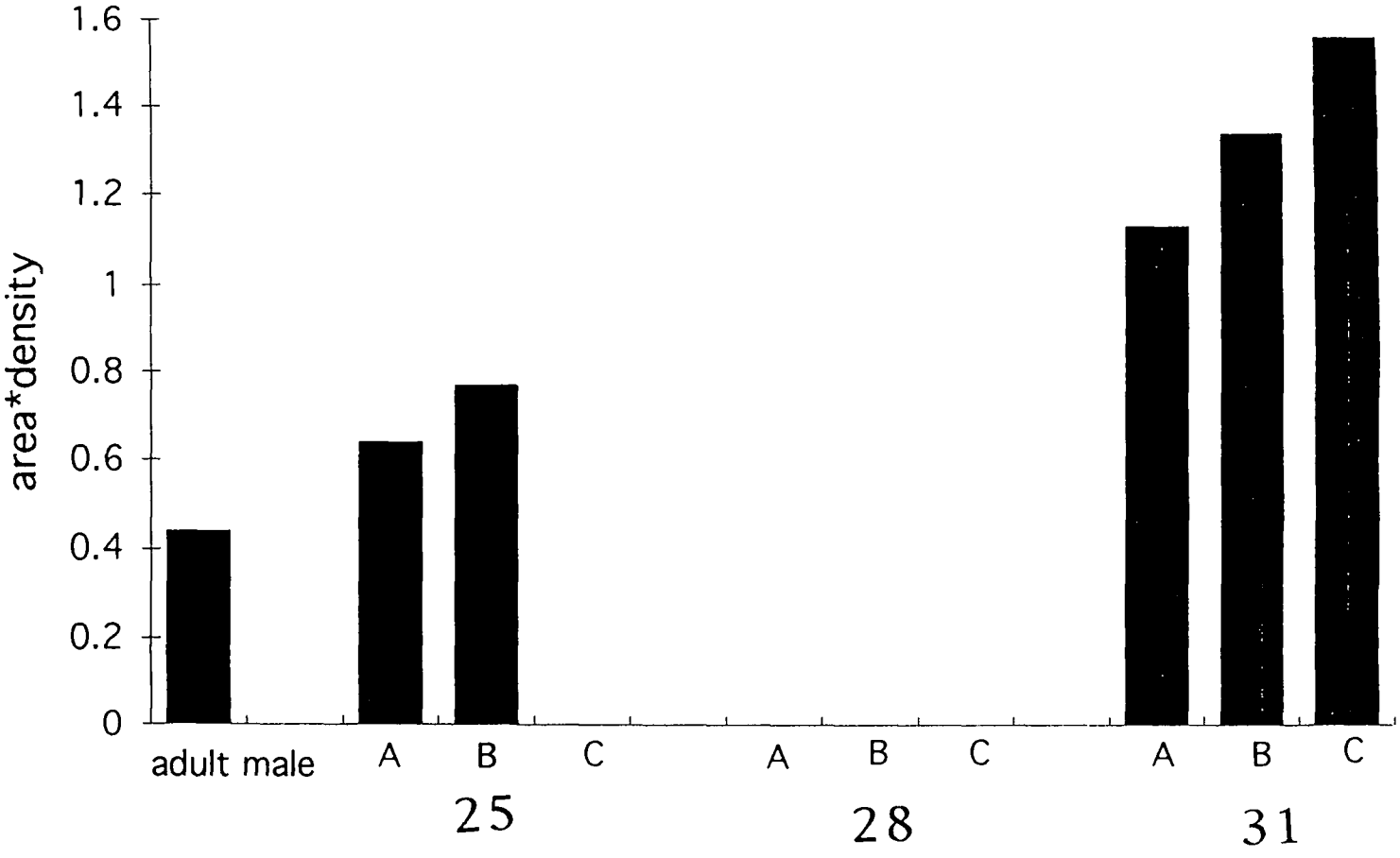
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
area*density
adult male
A
B
C
A
B
C
A
B
C
25
28
31

Figure 7

Fig. 8 Quantitation of FMO2 mRNA in lung from neonatal rabbit.

Samples of total RNA (4 μg) were applied to a slot blotter and the membrane hybridized to rabbit FMO2 cDNA (1142 bp) labeled with DIG by the random priming method. The data are expressed as area* density in arbitrary units after scanning densitometry as described in the materials and methods.
The number on the x-axis followed by pp indicates the days post-partum. The letters denote distinct litters and the numbered subscripts indicate individual neonates.

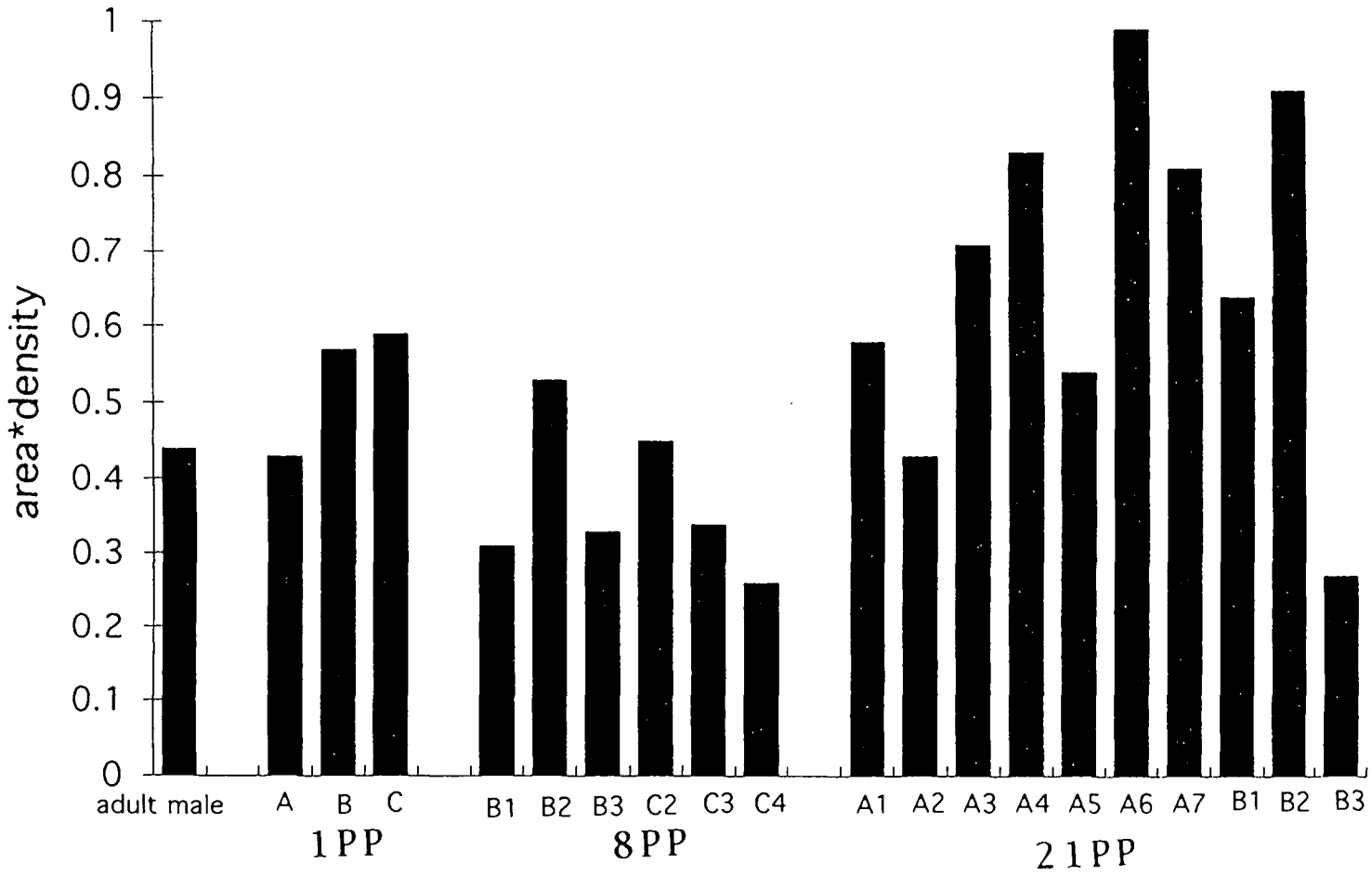
area*density
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
adult male
A
B
C
B1
B2
B3
C2
C3
C4
A1
A2
A3
A4
A5
A6
A7
B1
B2
B3
1PP
8PP
21PP

Figure 8

Fig. 9 Slot blot analysis of total RNA from rabbit fetal and neonatal lung, and control sample liver with cDNA encoding rabbit CYP2B4.

Samples of total RNA (4 μg) were applied to a slot blotter and membranes hybridized to CYP2B4 cDNAs labeled with DIG by the random priming method.
Top figures in (A) (B) (C) and (D) represent FMO2 cDNA hybridization.
(A) Lanes 1 through 12 (25A, 25B, 25C, 28A, 28B, 28C, 31A, 31B, 31C, 1ppA, 1ppB, 1ppC).
THe number (25, 28, or 31) indicates the day of gestation and the letter denotes that these were pooled fetuses from a single maternal rabbit. The number followed by pp indicates the days post-partum. The letters denote distinct litters.
(B) Lanes 1 through 3 (8ppB_1, B_2, B_3). (B) Lanes 4 through 5 (5 μg of liver RNA from 25A fetuses, adult male). (B) Lanes 6 through 11 (adult male lung RNA 0.2, 1, 2.5, 5, 10, and 15 μg).
(C) Lanes 1 through 12 (8pp C_2, C_3, C_4, 21pp A_1, A_2, A_3, A_4, A_5, A_6, A_7, B_1, B_2).
(D) Lane 1 (21pp B_3) (D) Lanes 4 and 5 (5 μg of liver RNA from 25A fetuses and adult male). (D) Lanes 6 through 11 (adult male lung RNA 0.2, 1, 2.5, 5, 10, and 15 μg)
The number followed by pp indicates the days post-partum. The letters denote distinct litters and the numbered subscripts indicate individual neonates.
Bottom figures show 16S rRNA hybridization for each.

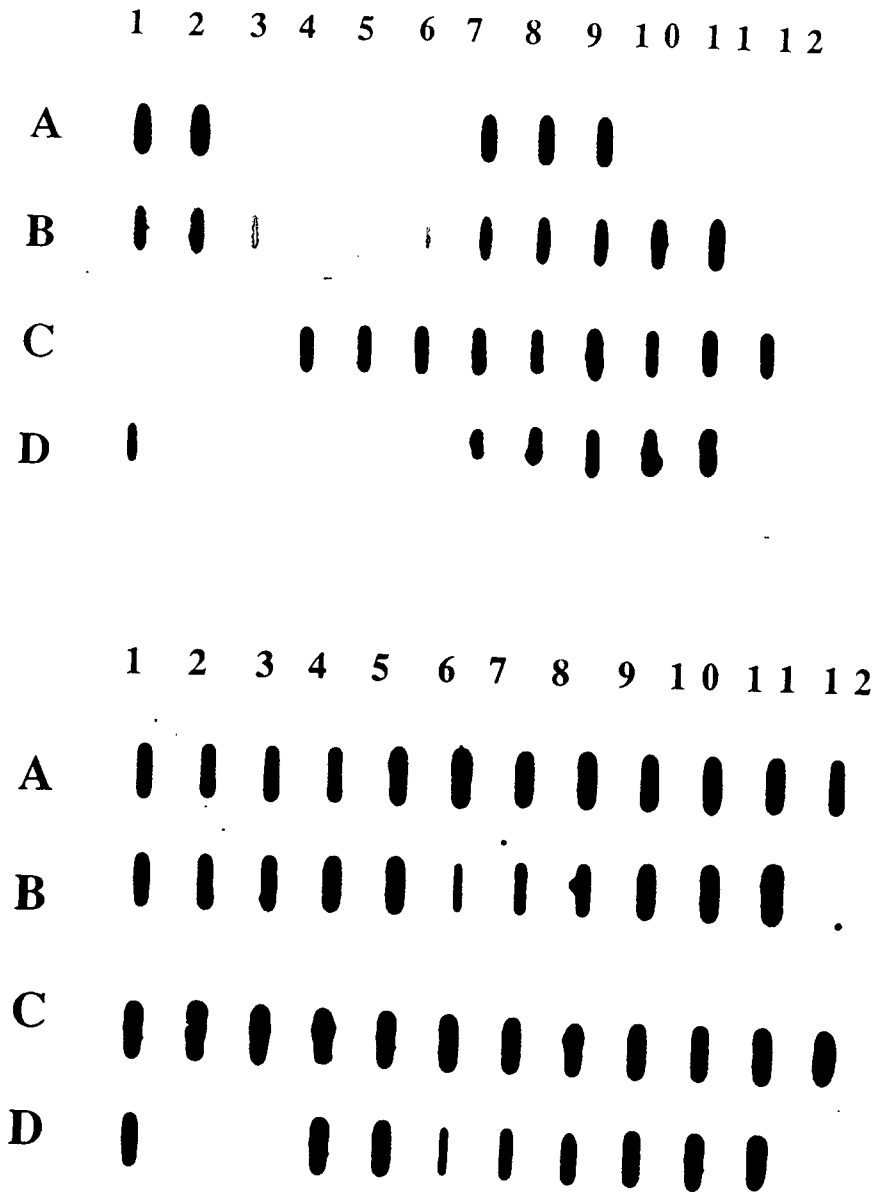

Figure 9

Fig. 10 Quantitation of CYP2B4 mRNA in lung from fetal rabbit.

Samples of total RNA (4 μg) were applied to a slot blotter and the membrane hybridized to rabbit CYP2B4 cDNA (2067 bp) labeled with DIG by the random priming method. The data are expressed as area* density in arbitrary units after scanning densitometry as described in the materials and methods.
The number on the x-axis indicates the day of gestation and the letter denotes that these were pooled fetuses from a single maternal rabbit.
25C, 28A, 28B and 28C had no detectable CYP2B4 mRNA.

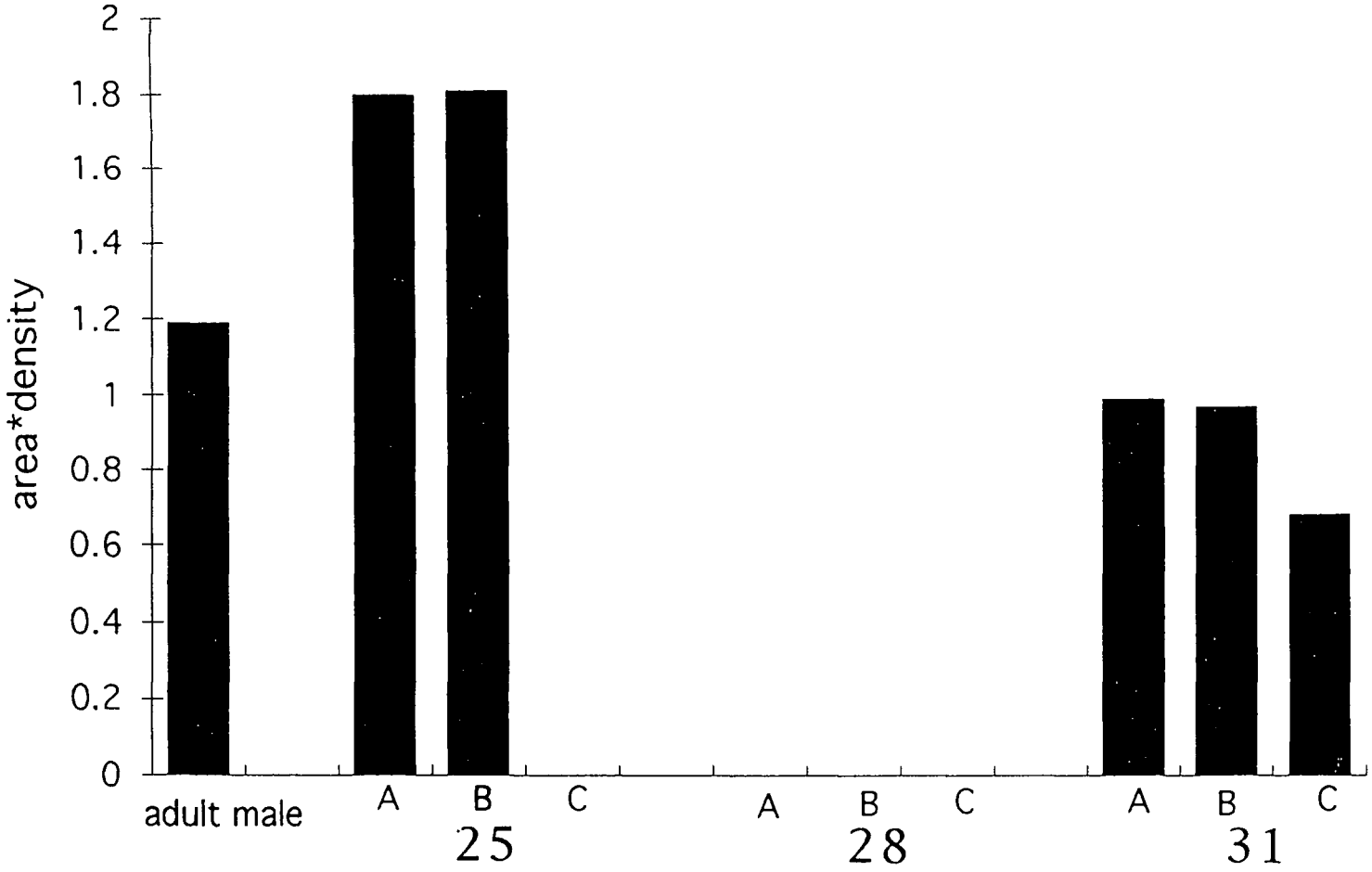
area*density
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
adult male
A
B
C
25
A
B
C
28
A
B
C
31

Figure 9

Fig. 11 Quantitation of CYP2B4 mRNA in lung from neonatal rabbit.

Samples of total RNA (4 μg) were applied to a slot blotter and the membrane hybridized to rabbit CYP2B4 cDNA (2067 bp) labeled with DIG by the random priming method. The data are expressed as area* density in arbitrary units after scanning densitometry as described in the materials and methods.
The number on the x-axis followed by pp indicates the days post-partum. The letters denote distinct litters and the numbered subscripts indicate individual neonates.

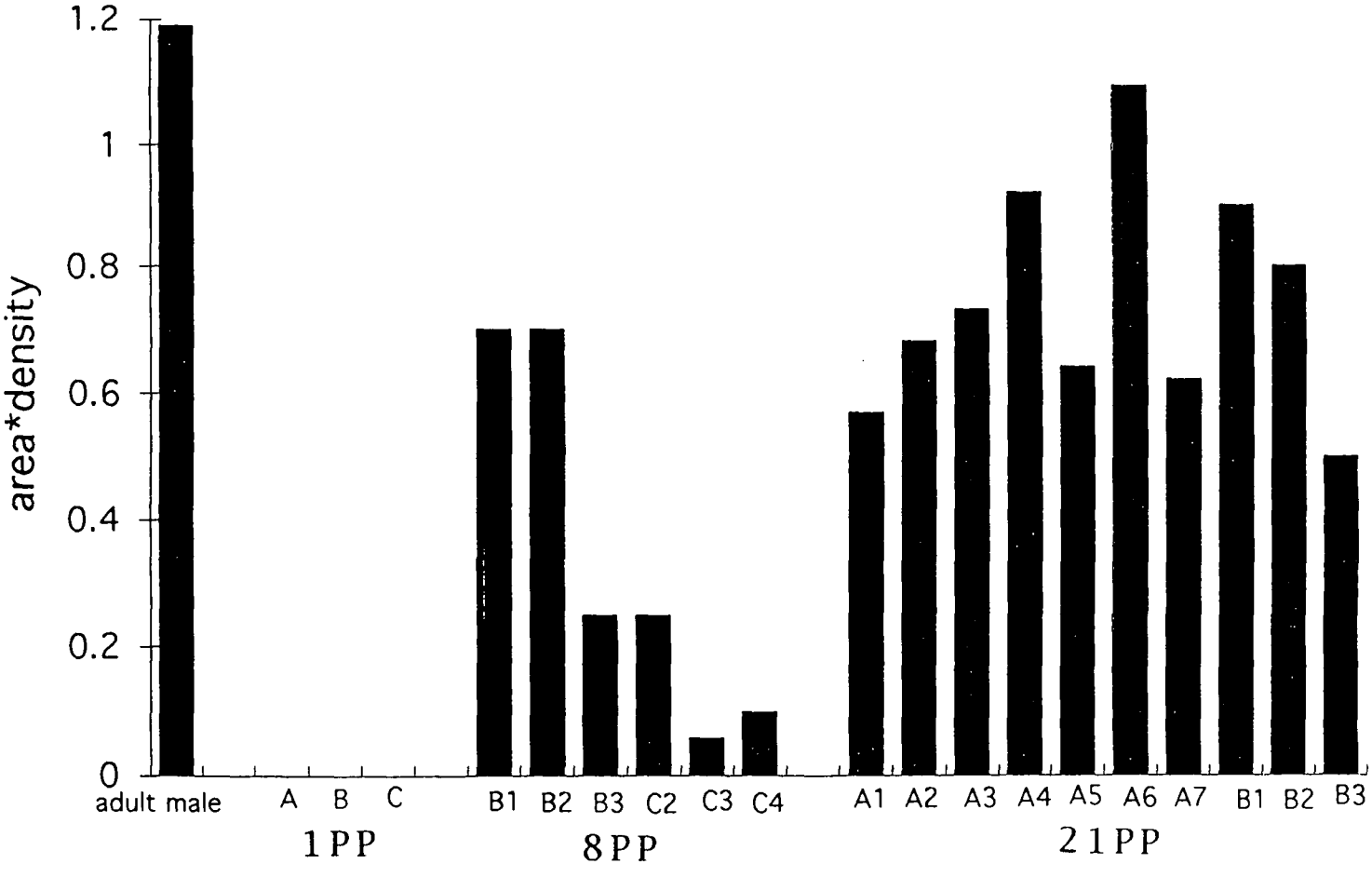
area*density
1.2
1
0.8
0.6
0.4
0.2
0
adult male
A
B
C
B1
B2
B3
C2
C3
C4
A1
A2
A3
A4
A5
A6
A7
B1
B2
B3
1PP
8PP
21PP

Figure 11

Fig. 12 Slot blot analysis of total RNA from rabbit fetal and neonatal lung, and control sample liver with cDNA encoding rabbit CYP4B1.

Samples of total RNA (4 μg) were applied to a slot blotter and membranes hybridized to CYP4B1 cDNAs labeled with DIG by the random priming method.
Top figures in (A) (B) (C) and (D) represent FMO2 cDNA hybridization.
(A) Lanes 1 through 12 (25A, 25B, 25C, 28A, 28B, 28C, 31A, 31B, 31C, 1ppA, 1ppB, 1ppC).
THe number (25, 28, or 31) indicates the day of gestation and the letter denotes that these were pooled fetuses from a single maternal rabbit. The number followed by pp indicates the days post-partum. The letters denote distinct litters.
(B) Lanes 1 through 3 (8ppB_1, B_2, B_3). (B) Lanes 4 through 5 (5 μg of liver RNA from 25A fetuses, adult male). (B) Lanes 6 through 11 (adult male lung RNA 0.2, 1, 2.5, 5, 10, and 15 μg).
(C) Lanes 1 through 12 (8pp C_2, C_3, C_4, 21pp A_1, A_2, A_3, A_4, A_5, A_6, A_7, B_1, B_2).
(D) Lane 1 (21pp B_3) (D) Lanes 4 and 5 (5 μg of liver RNA from 25A fetuses and adult male). (D) Lanes 6 through 11 (adult male lung RNA 0.2, 1, 2.5, 5, 10, and 15 μg)
The number followed by pp indicates the days post-partum. The letters denote distinct litters and the numbered subscripts indicate individual neonates.
Bottom figures show 16S rRNA hybridization for each.

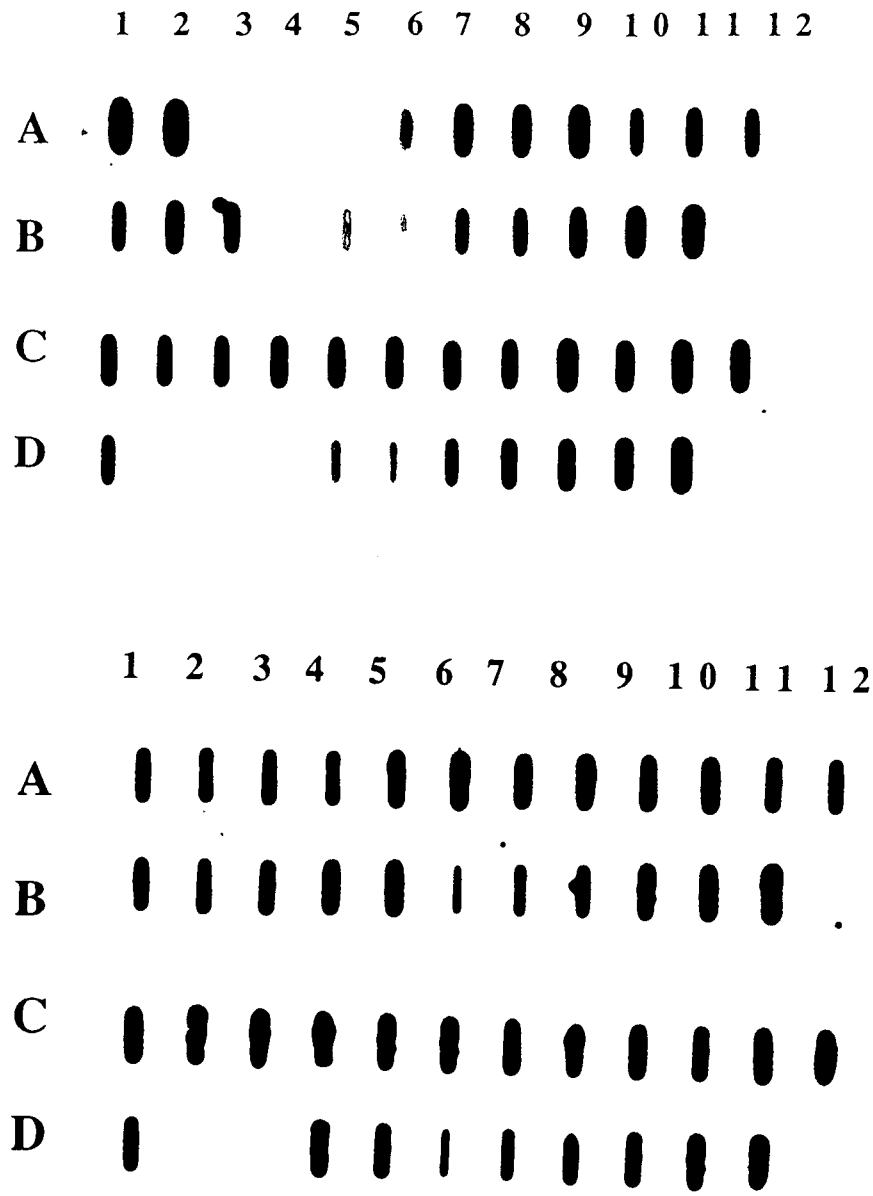

Figure 12

Fig. 13 Quantitation of CYP4B1 mRNA in lung from fetal rabbit.

Samples of total RNA (4 μg) were applied to a slot blotter and the membrane hybridized to rabbit CYP2B4 cDNA (1760 bp) labeled with DIG by the random priming method. The data are expressed as area* density in arbitrary units after scanning densitometry as described in the materials and methods.
The number on the x-axis indicates the day of gestation and the letter denotes that these were pooled fetuses from a single maternal rabbit.

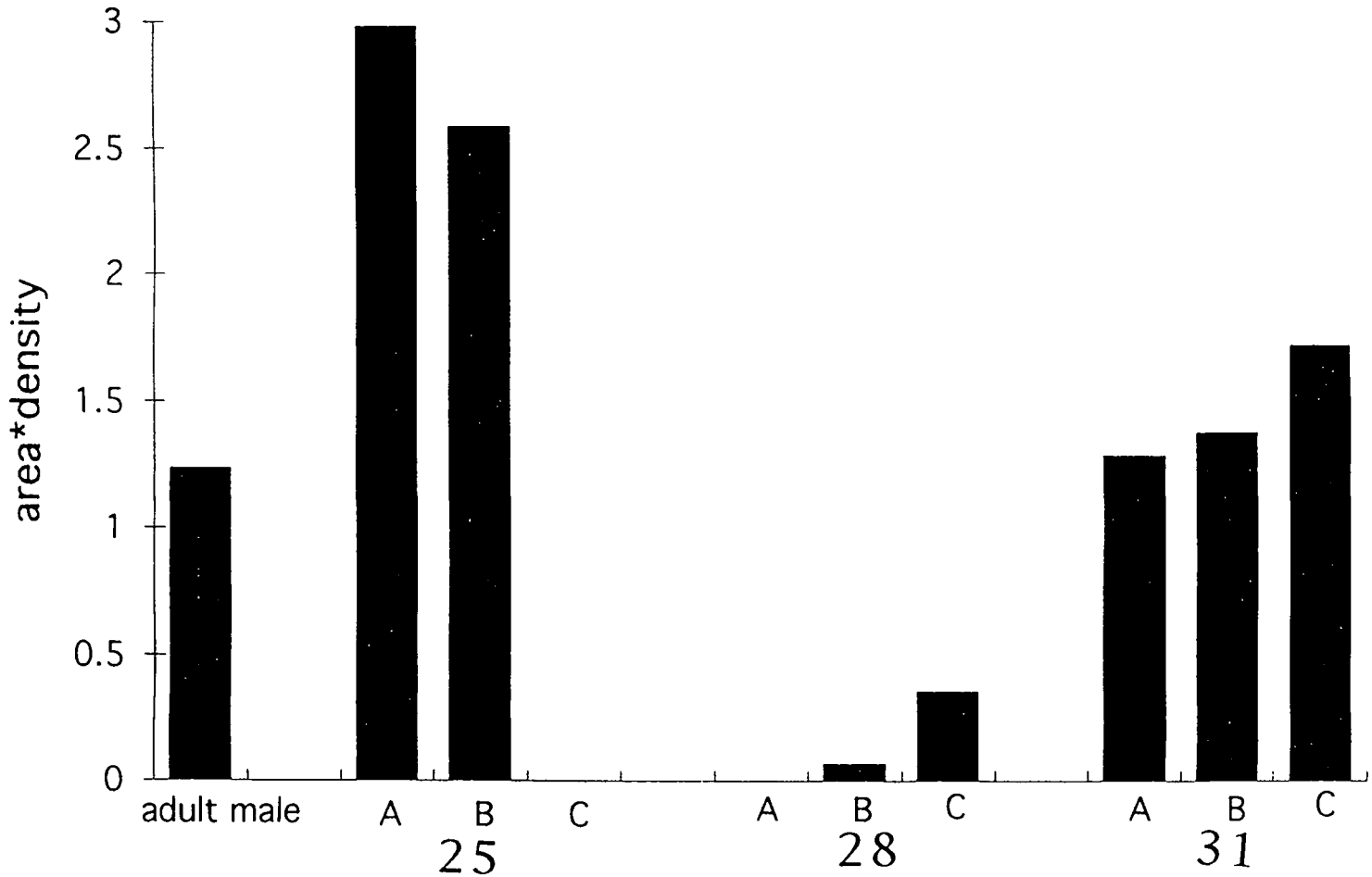

Figure 13

Fig. 14 Quantitation of CYP4B1 mRNA in lung from neonatal rabbit.

Samples of total RNA (4 μg) were applied to a slot blotter and the membrane hybridized to rabbit CYP4B1cDNA (1760 bp) labeled with DIG by the random priming method. The data are expressed as area* density in arbitrary units after scanning densitometry as described in the materials and methods.
The number on the x-axis followed by pp indicates the days post-partum. The letters denote distinct litters and the numbered subscripts indicate individual neonates.

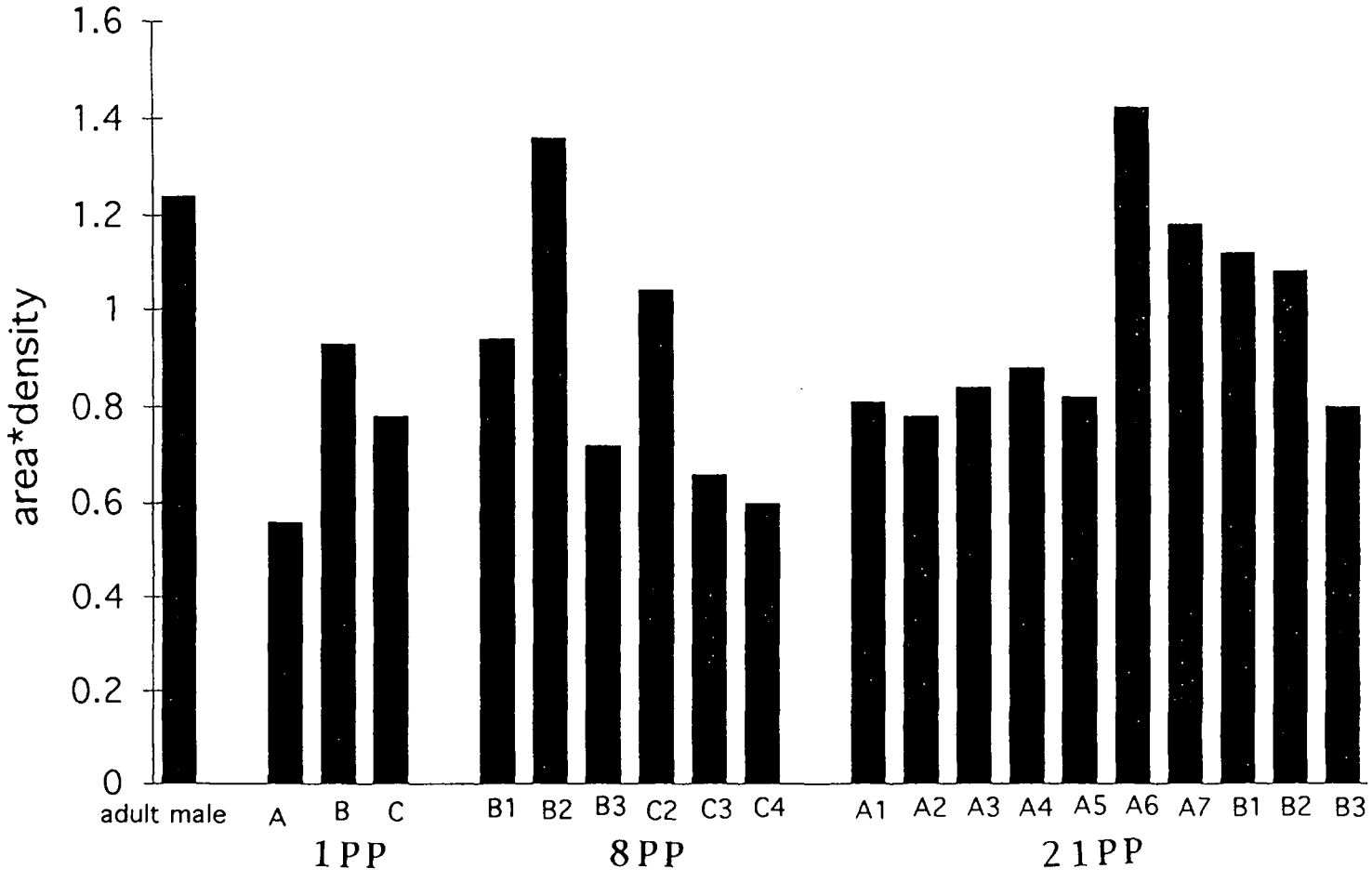
area*density
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
adult male
A
B
C
B1
B2
B3
C2
C3
C4
A1
A2
A3
A4
A5
A6
A7
B1
B2
B3
1PP
8PP
21PP

Figure 14

DISCUSSION

The cloning, sequencing and characterization of cDNA encoding rabbit liver (FMO1) and rabbit lung (FMO2) provided conclusive evidence for the existence of related but distinctly different FMO genes. (Gasser et al., 1990; Lawton et al., 1990). Recent studies have also indicated that expression of various FMO isoforms varies significantly with tissues and species. In rabbits, two distinct FMOs (FMO1 and FMO3) have been purified from liver (Ozols, 1990; Ozols, 1991) and a third isoform (FMO5) has been identified by cDNA cloning (Atta-Asafo-Adjei, 1993). In lung, FMO2 is expressed at much higher level than FMO1, FMO3 and FMO5 (Lawton et al., 1994; Atta-Asafo-Adjei, 1993; Lawton et al., 1990; Tynes and Philpot, 1987). In kidney, at least five FMOs are known to be expressed. Therefore, it is likely that a major contributor to differences in activity among tissues is the profile of the isoforms present. In this study, the application of northern and slot blot analyses provided both qualitative and quantitative profiles of steady-state mRNA levels and confirmed that mRNA are expressed in a tissue-specific manner for FMO1 and FMO2 in fetal and neonatal tissues.

tissue showed that FMO1 levels rose developmentally during pregnancy and following parturition. In fetal lung, FMO2 mRNA levels had reached that of adult male as early as the 25th day of gestation (the first time point measured). The biological significance of early developmental appearance of FMO in rabbit is presently unclear. However, in some fetal lung samples, there was no detectable FMO2 mRNA (fetuses from 25C, 28A, 28B, 28C rabbits). Similar mRNA distribution patterns of CYP2B4 and CYP4B1 also occurred on the 25^{th}, 28^{th} and 31^{st} day of gestation, compared to FMO2 levels. Comparison of the mRNA levels for FMO2 and CYP2B4, and CYP4B1 following parturition, generally also showed similar distribution patterns, except undetectable CYP2B4 mRNA on the first day postpartum. Whether individual differences, inhibition of gene transcription or some other factors led to non-expression of mRNA in these fetuses, still needs further study.

It is interesting to note the similar distribution patterns of mRNA expression among FMO2, CYP2B4 and CYP4B1 during gestation and postpartum. This indicates that FMO and P450 enzymes might cooperate in the metabolism of xenobiotics through most of the fetal and neonatal developmental stages. The relative contribution of FMO and P450s in the metabolism of xenobiotics has been studied recently. For instance, the primary pathway for nicotine metabolism in mammals involve iminium ion formation which is catalyzed mainly by CYP2B4 (Nakayama et al., 1982; Williams et al., 1990) The iminium ion could be regarded as a toxic metabolite because the nicotine-derived covalent binding to macromolecules is mediated by this pathway (Shigenaga et al., 1988). On the other hand, the highly

polar nicotine-N-oxide, produced by FMO, allows for rapid clearance into urine, representing detoxification (Duan et al., 1991). Further studies, such as immunoquantitation of FMO2 throughout gestation and development, and knowledge of the relative activity of FMO verse P450 in formation of metabolites derived from xenobiotics for which lung is target organ, will provide enhanced understanding of the role of FMO2 in toxicology.

The marked tissue-specific distribution of mRNA expression for FMO1 and FMO2 in fetus and neonate suggests that a major point of regulation might be transcription, and some unknown factors control the enzyme expression at the level of transcription. This issue could be addressed by nuclear run-on experiments to determine whether or not enhanced levels of FMO mRNA are due to increased rates of transcription. The whole process of transcription is quite complex. The activation or suppression of gene by regulatory proteins, DNA methylation (Razin and Cedar, 1991), the rate of transcription, processing of primary RNA, efficiencies of nucleocytoplasmic transport and stability of RNA in cytoplasma, all share responsibility for gene expression (Darnell and Darnell, 1982). The tissue-specific use of alternative promoters for gene transcription has recently been reported and hormones apparently play an important role in controlling alternative promoters in some genes (Maniatis et al., 1987; Mahendroo et al., 1993). Selection of alternative splicing pathways has been found to be another important regulatory step in expression of a number of genes and regulated splicing can function as on/off switch in gene expression (Andreadis et al., 1987). Isolation and characterization of FMO genes (genomic DNA cloning)

might be able to provide a better understanding for genomic DNA transcription apparatus such as promoter elements, distant enhancer elements or some other response elements regulating FMO gene expression.

In conclusion, the early development and tissue-specific expression patterns of FMO mRNA in rabbit fetus and neonate might have a significant role in the metabolism and target organ toxicity of xenobiotics during this critical and vulnerable time.

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