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Functional Cytochrome P4503A Isoforms in Human Embryonic Tissues: Expression During Organogenesis

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SUMMARY

Expression of functional cytochrome P450 (CYP) isoforms in human embryonic tissues was explored during organogenesis (days 50–60 of gestation) with substrate probes, inhibitor probes, and immunoprobes and by reverse transcription-polymerase chain reaction (PCR), cloning, and sequencing. Evidence was obtained for the presence of relatively high levels of one or more functional CYP3A isoforms in embryonic livers. This was manifested as relatively extensive hydroxylation of (*R*)-warfarin at carbon 10 and as triacetyloleandomycin-inhibited *O*-debenzylation of benzyloxyresorufin when human embryonic hepatic microsomal fractions were used as enzyme sources. Immunoblots with anti-CYP3A4 antibody exhibited a strong signal in embryonic hepatic tissues but, in contrast, indicated very low or negligible CYP3A levels in human embryonic lung, kidney, heart, adrenal, and brain tissues. To explore expression of individual members of the CYP3A subfamily in human embryonic hepatic tissues at this early gestational stage, CYP3A cDNA was generated by reverse transcription, amplified by PCR, cloned, and sequenced. Oligonucleotide primers used for PCR were designed to flank target sequences unique to *CYP3A* but also common to all human *CYP3A* subfamily members for which GenBank nucleotide sequence information was available (*CYP3A3*, *CYP3A4*, *CYP3A5*, *CYP3A5P*, and *CYP3A7*). Sequencing data indicated that plasmids in 58 of 59 recombinant positive colonies contained an insert with a sequence identical to that present in *CYP3A7* cDNA and the plasmid of only one colony contained an insert with a sequence identical to that present in *CYP3A5* cDNA. No evidence was found for expression of *CYP3A3* or *CYP3A4*. Thus, during organogenesis, human embryonic hepatic tissues express primarily CYP3A7 and are capable of significant CYP3A7-catalyzed xenobiotic monooxygenation during this very early stage of gestation.

During the period of human organogenesis, which extends roughly from day 18 to day 60 of gestation, the prenatal organism (conceptus) is referred to as an embryo rather than as a fetus. Organogenesis is a very early period of development, during which developing concepti are widely held to be most sensitive to the teratogenic/dysmorphogenic effects of exogenous chemicals. Although the CYP enzyme system is strongly implicated in the bioactivation of such xenobiotics, very little information is available on the functional CYP complement of human embryonic tissues. In contrast, expression of relatively high levels of enzymically functional, xenobiotic-biotransforming CYPs in human conceptal tissues during the fetal period of gestation (roughly day 60 of gestation until term) has been recognized for more than two decades (1–3).

The identification of specific individual CYP isoforms in human fetal hepatic tissues, however, has been a relatively recent occurrence. Possibly the most striking finding has been that a member of the CYP3A subfamily, now known to be CYP3A7, is expressed at relatively high levels in human fetal livers but reportedly not in the normal livers of adult humans (4, 5). Also very notable is that no CYP3A isoform has been reported to be detectable in the fetal livers of common experimental animals, such as rats, mice, and rabbits, except after induction or just before parturition (6, 7). Kitada *et al.* (8) have reported the purification of four forms of CYP (designated by those authors as P450HFLa, P450HFLb, P450HFLc, and P450HFLd) from human fetal livers. Evidence indicates that P450HFLa is CYP3A7, that P450HFLb is a member of the CYP1A subfamily, that P450HFLc is another member of the CYP3A subfamily (perhaps CYP3A5), and that P450HFLd is an isoform of unknown identity (8). Several other groups of investigators also have reported evidence for the expression of multiple CYP isoforms in human fetal hepatic tissues (9–13).

Numerous questions arise as a result of the observations in human fetal hepatic tissues. Among these are questions pertaining to whether functional CYP isoforms would be significantly expressed in human embryonic tissues during the period of organogenesis, the levels at which individual CYP isoforms

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ABBREVIATIONS: CYP or P450, cytochrome P450; RT, reverse transcription; PCR, polymerase chain reaction; TAO, triacetyloleandomycin; G6P, glucose-6-phosphate; BZROD, benzyloxyresorufin *O*-debenzylase; AMV, avian myeloblastosis virus; SDS, sodium dodecyl sulfate.

would be expressed in specific embryonic tissues, and the substrate specificities of any embryo-specific isoforms. Recent investigations from this laboratory (14, 15) have indicated that multiple functional (enzymically active) CYP isoforms can also be expressed in human embryonic tissues during this period but, as yet, none have been rigorously identified. The purpose of these investigations, therefore, was to determine whether CYPs of the 3A subfamily are significantly expressed in human embryonic tissues during organogenesis, whether such CYPs are functionally active in terms of catalysis of xenobiotic monooxygenation(s), in which tissues significant expression might occur, and, finally, which specific CYP3A isoforms can be identified. In addition, some of our earlier investigations (14) suggested that functional CYP3A might not be significantly expressed in human embryonic tissues during this early period of gestation. This was based on the observation that TAO, a specific CYP3A inhibitor, failed to significantly inhibit human embryonic BZROD activities. Because this was somewhat unexpected, in view of the relatively high levels of expression of enzymically functional CYP3A7 in human fetal hepatic tissues, we wished to examine this aspect in some detail. In light of the reported capacities of human fetal CYPs to catalyze the bioactivation of various promutagens, procarcinogens, prodysmorphogens, etc. (8, 16–19), resolution of these questions is clearly of primary importance for studies of embryonic development and organogenic processes. Results obtained in this study indicate that, during organogenesis, enzymically functional CYP3A7 is expressed at relatively high levels in human embryonic hepatic (but not various extrahepatic) tissues between days 50 and 60 of gestation and that other CYP3A isoforms appear to be expressed minimally or not at all in the same tissues.

Materials and Methods

Chemicals and reagents. Optically pure (*R*)- and (*S*)-warfarin enantiomers, metabolites, and deuterated analogues were obtained from the various sources described by Rettie *et al.* (20). 7-Benzyloxyphenoxazone (commonly referred to, and referred to here, as benzyloxyresorufin) was synthesized in our laboratory according to methods described by Mayer *et al.* (21). Resorufin and dicoumarol were purchased from Aldrich Chemical Co. (Milwaukee, WI). NADPH, G6P, and G6P dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO). Resorufin and benzyloxyresorufin were purified (>99.5%) according to methods described by Klotz *et al.* (22). TAO was received as a generous gift from the Pfizer Laboratories (New York, NY). Purified CYP3A4 (16.2 nmol/mg of protein) and polyclonal antibody raised against human CYP3A4 were generously supplied by Dr. Kenneth E. Thummel, University of Washington. Spectrophotochemical grade dimethylsulfoxide was purchased from the J. T. Baker Chemical Co. (Phillipsburg, NJ). All other chemicals used were of the highest purity commercially available.

Human embryonic tissues. Hepatic, adrenal, renal, pulmonary, cardiac, and brain tissues from human embryos and human fetal hepatic tissues were procured through the Central Embryology Laboratory (Department of Pediatrics) of the University of Washington (Seattle, WA). All procedures involving handling and assay of these tissues were in accordance with the guidelines of the Human Subjects Review Committee at the same institution. Tissues were obtained immediately after surgical procedures (e.g., dilatation and curettage), placed on ice, and delivered to the laboratory within 3–4 hr. Gestational ages of all embryonic specimens obtained were 50–60 days, as estimated from measurements of foot lengths. Human fetal hepatic tissues from specimens with gestational ages of 72–152 days, as estimated from measurements of crown-rump lengths, were obtained for purposes of standardization and comparison. Upon arrival in the laboratory, tissues were immediately frozen and stored at −70°.

Analyses of enzymically catalyzed reactions. Because of the very small quantities of human embryonic tissues obtainable between 50 and 60 days of gestation, it was necessary to pool these tissues from several embryos. Thus, pooled tissues from four to 10 embryos were used unless otherwise specified. Tissues were thawed and homogenized manually in a glass homogenizing vessel (Potter) with a ground glass pestle, in 3 volumes of ice-cold 0.1 M potassium phosphate buffer, pH 7.4. The resultant homogenate was centrifuged at 600 × *g* for 5 min and an aliquot of the supernatant fraction was saved for subsequent analyses. The remainder of the 600 × *g* supernatant fraction was centrifuged at 104,000 × *g* for 1 hr, and the 104,000 × *g* supernatant fraction (designated as the cytosolic fraction) was likewise saved for subsequent analyses. The 104,000 × *g* sediment was washed by resuspension in 5–6 ml of 0.1 M potassium phosphate buffer, pH 7.4, and recentrifugation at 104,000 × *g* for 1 hr. The resultant sediment was resuspended in a volume of 0.1 M potassium phosphate buffer, pH 7.4, equivalent to the original volume of the cytosolic fraction and was centrifuged at 8000 × *g* for 20 min, to eliminate larger fragments of particulate aggregates that could interfere with the continuous fluorimetric assay. The resultant 8000 × *g* supernatant fraction was designated as the microsomal fraction and was utilized in subsequent BZROD analyses. In certain experiments with fetal livers, the hepatic tissues were homogenized and centrifuged at 8000 × *g* for 20 min, and the supernatant fraction was used as an enzyme source without further processing. Centrifugation of the fetal hepatic 8000 × *g* supernatant fraction at 104,000 × *g* for 1 hr yielded supernatant (cytosolic fraction) and sediment (microsomal fraction) that also were used as enzyme sources.

BZROD activities were assessed fluorimetrically according to slightly modified methods originally described by Burke *et al.* (23). Unless otherwise specified, incubation cuvettes contained 0.04–0.3 mg of protein (0.1 ml), 0.01 mM benzyloxyresorufin dissolved in 0.01 ml of dimethylsulfoxide, 1.0 mM NADPH, an NADPH-regenerating system consisting of 5.0 mM G6P and 1 unit of G6P dehydrogenase, 0.01 mM dicoumarol, and sufficient Tris·HCl buffer (0.1 M, pH 7.8) to yield a total volume of 1.0 ml. After a base-line was obtained, reactions were initiated by additions of benzyloxyresorufin. Temperatures of the reaction mixtures were maintained at 37° during measurements of resorufin generation. Reactions were monitored continuously as a function of time, with a Farrand recording fluorimeter with excitation and emission wavelengths of 530 and 585 nm, respectively. Generation of resorufin was measured for at least 8 min and the slopes of the lines for the first 3 min were used to calculate initial rates of the reactions. The extent of fluorescence increase was calibrated against a known concentration of purified resorufin standard in each assay. The limit of detection was 0.1 pmol/min.

For analyses of biotransformation of (*R*)- and (*S*)-warfarin, reaction vessels contained 1 mM NADPH, 1 mM (*R*)- or (*S*)-warfarin as substrate, 0.1–0.2 mg of protein, and sufficient 0.1 M potassium phosphate buffer, pH 7.4, to yield a total volume of 1.0 ml. Incubations were processed in glass vials at 37° for 2 hr under oxygen/nitrogen (20:80). Reactions were initiated by addition of NADPH and were terminated by additions of 0.6 ml of ice-cold acetone. Hydroxylated metabolites of warfarin were extracted, derivatized, and analyzed using a VG 707OH double-focussing mass spectrometer operated in the selected ion-monitoring mode, as described previously (Ref. 24 and cited references therein). Enzyme activities were expressed as picomoles of metabolite generated/time of incubation/milligram of protein. Protein values were determined by the method of Lowry *et al.* (25).

Immunoblotting. Immunoblots were performed with polyclonal antibodies raised against human CYP3A4 (26) and polyclonal antibodies raised against adult rat hepatic CYP1A1/2, -2B1/2, and -2C11, produced as described earlier (14, 15, 27, 28). Specificity and other characteristics of the anti-CYP3A antibody have been described in

detail by Kharasch and Thummel (26), and those of the other antibodies are described in the references cited (14, 15, 27, 28). Aliquots of embryonic or fetal homogenate subfractions were applied to 7.5% or 10.0% SDS-polyacrylamide gels and further processed for immunoblotting with standard protocols as described previously (27). Immunoreactive bands were visualized with high sensitivity Enzygraphic Web (International Biotechnologies, New Haven, CT). Additional details are provided in the legends to Figs. 1 and 2.

RNA preparation, first-strand cDNA synthesis, and PCR amplification of cDNA. For these experiments, human embryonic or fetal hepatic tissues were homogenized as described by Yang *et al.* (28). Total RNA was extracted from conceptal hepatic homogenates as described by Chomczynski and Sacchi (29), and mRNA was isolated with a Poly(A)$^+$ Quick mRNA purification kit (Stratagene, La Jolla, CA). RT of human conceptal hepatic mRNA was performed with oligo(dT)$_{15}$ (Promega, Madison, WI) and AMV reverse transcriptase (Life Sciences, St. Petersburg, FL), as described earlier (28). The PCR sense primer utilized was 5′-CTGGATCCTAGCTGAGGATGAAGAA-TGG-3′ and the antisense primer was 5′-CTGGATCCGTGGATTG-TTGAGAGAGTCG-3′ (underlined sequences indicate *Bam*HI sites). These primers were designed to target cDNA sequences unique to human CYP3A DNA (*CYP3A*) forms but also common to all known genes of the human *CYP3A* subfamily (*CYP3A3*, *CYP3A4*, *CYP3A5*, *CYP3A5P*, and *CYP3A7*) for which information was available from the GenBank/EMBL database (using the Genetics Computer Group package). These sequences included nucleotides 419–658 (*CYP3A3*, GenBank/EMBL accession number D00003), 449–688 (*CYP3A4*, accession number M18907), 446–685 (*CYP3A5*, accession number J04813), 670–918 (*CYP3A5P*, accession number L26985), and 362–601 (*CYP3A7*, accession number D00408). After RT, PCR was performed in a final volume of 50 μl, containing 10 mM Tris·HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1 mM $MgCl_2$, 0.2 mM levels of each deoxynucleoside triphosphate, 25 pmol each of sense and antisense primers, 5 μl of cDNA, and 2.5 units of *Thermus aquaticus* polymerase (Promega). After the addition of 100 μl of mineral oil, the reaction mixtures were heated to 93° for 5 min and immediately cycled 30 times in a Perkin-Elmer Cetus thermocycler. The amplification cycle profile included denaturation at 93° for 1 min, annealing at 54° for 1.5 min, and extension at 72° for 1 min.

Cloning and sequencing of RT-PCR-amplified cDNA. The cDNA band on the 2% low-melting point agarose gel was excised and the cDNA was purified according to methods described by Qian and Wilkinson (30). Briefly, the slice of low-melting point agarose gel containing the cDNA was placed in a microcentrifuge tube and 3 volumes of 1× TE buffer (10 mM Tris·HCl, 1 mM EDTA, pH 8.0) were added. The tube was incubated at 70° for 5 min to melt the agarose, with brief vortexing during the incubation. The sample was then quick frozen at −70° for at least 5 min. It was then permitted to thaw and was centrifuged at 16,000 × *g* for 1–2 min. The agarose-free cDNA in the supernatant fraction was precipitated with ethanol and then ligated into Bluescript vector (SK−), which had been previously cut with *Bam*HI and treated with calf intestinal alkaline phosphatase. Sequencing was performed on both strands with Sequenase 2 (United States Biochemical Life Sciences Co., Cleveland, OH), in accordance with the manufacturer's instructions.

Results

Investigation of the capacity of human embryonic hepatic tissues to catalyze the conversion of (*R*)- and (*S*)-warfarin to various hydroxylated metabolites yielded results presented in Table 1. For both *R*- and *S*-enantiomers, the 10-hydroxylated metabolite was strongly dominant, with generation of 10-hydroxy-(*R*)-warfarin exceeding that of 10-hydroxy-(*S*)-warfarin by a factor of approximately 4 under the same reaction conditions. Formation of 4′- and 6-hydroxy metabolites was also easily detectable, but conversion of both *R*- and *S*-enantiomers to 7- and 8-hydroxy metabolites was near the limits of detectability. From the perspective of extensive past investigations with warfarin as a CYP probe substrate (e.g., 20, 24, 31–34), the results indicated that an enzymically active CYP3A isoform was expressed at very easily detectable levels in human embryonic hepatic tissues, but this concept was not supported by previous data (14) indicating a lack of detectable, TAO-inhibitable, BZROD activity and leading to the opposite conclusion. Therefore, we further investigated the BZROD activity of human embryonic hepatic preparations (Table 2). Surprisingly, these investigations revealed that >70% of the BZROD activity measurable in 600 × *g* (for 5 min) supernatant fractions of human embryonic hepatic homogenates was localized in a membrane-free subfraction (designated the cytosolic fraction) and also that the BZROD activity in the cytosolic fraction was not inhibited by 600 μM concentrations of TAO. However, BZROD activity of the microsomal fraction of the same homogenates, under the same preincubation and incubation conditions, was markedly inhibited (~84% inhibition) by 600 μM TAO. Similar phenomena also were observed in experiments with human fetal livers at a relatively late stage of gestation (Table 2). Direct comparisons of human embryonic and fetal livers were made because it is well documented that functional 3A isoforms are expressed in prenatal human livers during the later (fetal) stage of gestation but little or no corresponding data have appeared for the embryonic period. Again, inhibition by TAO of BZROD activities in fetal hepatic tissue preparations (8000 × *g*, for 20 min, supernatant fractions) containing the cytosolic fraction was very weak and statistically insignificant, whereas inhibition of the same activity in the microsomal fraction was profound (~78%).

Comparisons of human embryonic hepatic preparations (days 50–60 of gestation) (Table 1) with human fetal hepatic preparations (day 152 of gestation) (Table 3) for generation of hydroxylated (*R*)- and (*S*)-warfarin metabolites indicated that human embryonic and fetal hepatic tissue preparations generated very similar (although not identical) patterns of metabolites. Insofar as measurements of warfarin hydroxylation were concerned, the presence of the fetal hepatic cytosolic fraction did not appear to markedly affect the overall pattern of microsomal metabolite formation [as judged from comparisons of 8000 × *g*, for 20 min, supernatant fractions versus 104,000 × *g*, for 1 hr, sediment (microsomal) fractions] (Table 3), although

TABLE 1

Hydroxylation of (*R*)- and (*S*)-warfarin enantiomers in incubation flasks containing human embryonic hepatic tissue as enzyme source

Results are expressed as mean nmol of metabolite generated/incubation vessel, from replicate determinations. Vessels contained 0.22 mg of protein and were incubated with 1.0 mM substrate at 37° in a 20% oxygen atmosphere for 2 hr. The enzyme source was the 600 × *g* for 5 min supernatant fraction of homogenates of pooled human embryonic livers from days 50–60 of gestation (see Materials and Methods.

Metabolite	Amount produced		*R*/*S* ratio
	(*R*)-Warfarin	(*S*)-Warfarin	
	nmol/vessel		
10-Hydroxy	3.54	0.93	3.8
8-Hydroxy	0.02	ND[a]	
7-Hydroxy	0.03	0.04	0.8
6-Hydroxy	0.29	0.54	0.5
4′-Hydroxy	0.55	0.08	6.9

[a] ND, quantities generated were below the level of detectability (<0.01 nmol).

TABLE 2

BZROD activities in subfractions of human embryonic or fetal hepatic homogenates and inhibition by TAO

Values are means ± standard deviations of specific activities from three or four experiments with pooled human embryonic livers from days 50–60 of gestation (see Materials and Methods) or with human fetal liver from day 152 of gestation.

Homogenate fraction	Inhibitor	BZROD activity
		pmol/mg of protein/min
Embryonic		
600 × *g* supernatant[a]	None	6.8 ± 1.9
600 × *g* supernatant	TAO (120 μM)	6.6 ± 1.3
600 × *g* supernatant	TAO (600 μM)	6.1 ± 2.1
Cytosolic fraction[b]	None	8.2 ± 1.5
Cytosolic fraction	TAO (600μM)	8.0 ± 1.9
Microsomal fraction[c]	None	1.9 ± 0.4
Microsomal fraction	TAO (600 μM)	0.3 ± 0.1
Fetal		
8000 × *g* supernatant[d]	None	6.7 ± 1.4
8000 × *g* supernatant	TAO (120 μM)	5.9 ± 1.8
8000 × *g* supernatant	TAO (600 μM)	5.8 ± 1.6
Microsomal fraction[e]	None	8.8 ± 2.1
Microsomal fraction	TAO (120 μM)	2.1 ± 0.6
Microsomal fraction	TAO (600 μM)	1.9 ± 0.8

[a] Human embryonic hepatic homogenates (see Materials and Methods) were centrifuged at 600 × *g* for 5 min.

[b] The 600 × *g* supernatant fractions were centrifuged at 104,000 × *g* for 1 hr. The resultant supernatant was designated as the cytosolic fraction.

[c] The 600 × *g* supernatant fractions were centrifuged at 104,000× *g* for 1 hr. The resultant sediment was washed by resuspension in potassium phosphate buffer (0.1 M, pH 7.4) and recentrifugation at 104,000 × *g* for 1 hr. The resultant sediment was resuspended in a volume of potassium phosphate buffer (0.1 M, pH 7.4) equivalent to that of the original 600 × *g* for 5 min) supernatant fraction and was centrifuged at 8000 × *g* for 20 min, and the resultant supernatant fraction was designated as the microsomal fraction.

[d] Human fetal hepatic homogenate (see Materials and Methods) was centrifuged at 8000 × *g* for 20 min.

[e] The 8000 × *g* supernatant fraction was centrifuged at 104,000 × *g* for 1 hr. The resultant sediment was resuspended in a volume of potassium phosphate buffer (0.1 M, pH 7.4) equivalent to that of the original 8000 × *g* (for 20 min) supernatant fraction and was designated as the microsomal fraction.

TABLE 3

Hydroxylation of (*R*)- and (*S*)-warfarin enantiomers in incubation flasks containing human fetal hepatic microsomes as enzyme source

Results are expressed as mean nmol of metabolite generated/incubation vessel, from replicate determinations. Vessels were incubated with 1.0 mM substrate at 37° in a 20% oxygen atmosphere for 120 min, with 1.9–3.0 mg of protein. Homogenate subfractions were from human fetal liver at day 152 of gestation.

Metabolite	Subfraction	Amount produced	
		(*R*)-Warfarin	(*S*)-Warfarin
		nmol/vessel	
10-Hydroxy	Microsomal fraction[a]	12.45	3.20
8-Hydroxy	Microsomal fraction	0.13	0.04
7-Hydroxy	Microsomal fraction	0.15	0.12
6-Hydroxy	Microsomal fraction	0.91	2.92
4′-Hydroxy	Microsomal fraction	2.57	1.55
10-Hydroxy	8000 × *g* supernatant[b]	3.93	1.11
8-Hydroxy	8000 × *g* supernatant	0.03	ND[c]
7-Hydroxy	8000 × *g* supernatant	0.04	0.05
6-Hydroxy	8000 × *g* supernatant	0.41	1.23
4′-Hydroxy	8000 × *g* supernatant	0.66	0.32

[a] See Table 2, footnote *e*.

[b] See Table 2, footnote *d*.

[c] ND, quantities generated were below the level of detectability (<0.01 nmol).

somewhat greater than expected quantities of the 6-hydroxylated metabolite of (*S*)-warfarin appeared when 8000 × *g* (for 20 min) supernatant fractions were used as enzyme sources. Ratios of both 6-hydroxy-(*S*)-warfarin and 4′-hydroxy-(*S*)-warfarin to 10-hydroxy-(*R*)-warfarin were somewhat higher at the later (fetal) stage of gestation, but this latter observation must be regarded as only preliminary.

Immunoblotting experiments performed with anti-CYP3A4 antibody (Fig. 1) indicated that readily detectable quantities of anti-CYP3A4-immunoreactive protein were present in the microsomal fractions of human embryonic hepatic homogenates between days 50 and 60 of gestation. Attempts to detect analogous immunoreactive protein in several other human embryonic tissues with the same antibody, at the same stage of gestation and with equivalent quantities of embryonic protein applied to the gels (Fig. 2), however, yielded only negative results. Negative immunoblots were also obtained with human embryonic adrenal tissues, which were assayed in a separate experiment (data not shown). Immunoreactive proteins did appear routinely in cerebral and pulmonary preparations and occasionally in renal embryonic preparations but exhibited molecular masses (~150 kDa) that clearly were not common to CYP proteins, particularly CYP3A forms. In addition, immunoblotting experiments with anti-CYP1A1/2, anti-CYP2B1/2, and anti-CYP2C11 antibodies yielded only negative results with each of the six human embryonic tissues investigated, with roughly equivalent quantities of protein applied to the gels (data not shown). In each case, immunoblotting with adult rat liver microsomes as controls yielded very strong signals of appropriate molecular mass. The focus in subsequent experiments, therefore, was on the identity of the CYP3A isoform(s) in human embryonic hepatic tissues.

PCR amplification of cDNA reverse transcribed from human embryonic hepatic mRNA with primers designed to target sequences unique to human *CYP3A* but common to all known genes or pseudogenes of the human *CYP3A* subfamily (*CYP3A3*, *CYP3A4*, *CYP3A5*, *CYP3A5P*, and *CYP3A7*) indicated that CYP3A mRNA was expressed in both human embryonic and fetal livers, with distinct signals appearing as the expected bands (~250 base pairs, including the *Bam*HI sites) (see legend to Fig. 3) on ethidium bromide-stained agarose gels (Fig. 3). Cloning and sequencing of the amplified cDNAs indicated that plasmids in 58 of the 59 colonies obtained contained inserts with sequences identical to segments present in *CYP3A7* (at positions 362–601) and the plasmid in the only other colony contained an insert with a sequence identical to a segment present in *CYP3A5* (at positions 446–685) (Fig. 4).

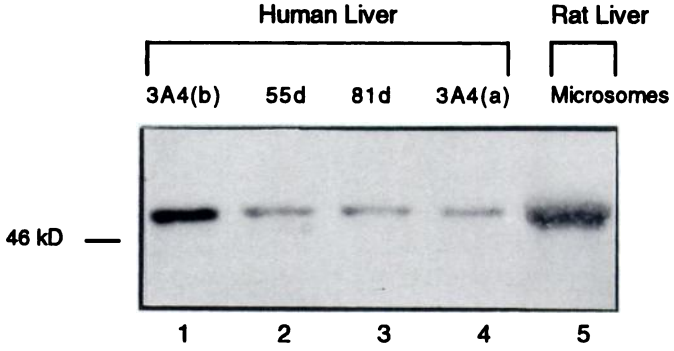

Fig. 1. Immunoblots of 10% SDS-polyacrylamide gels. *Lane 1*, purified CYP3A4 (0.12 μg of protein); *lane 2*, human embryonic hepatic homogenate 600 × *g* (for 5 min) supernatant fraction at gestational day 55 (0.05 mg of protein); *lane 3*, human fetal hepatic homogenate 600 × *g* (for 5 min) supernatant fraction at gestational day 81 (0.03 mg of protein); *lane 4*, purified CYP3A4 (0.03 μg of protein); *lane 5*, hepatic microsomal fraction prepared from Aroclor-treated adult male rats (0.05 mg of protein).

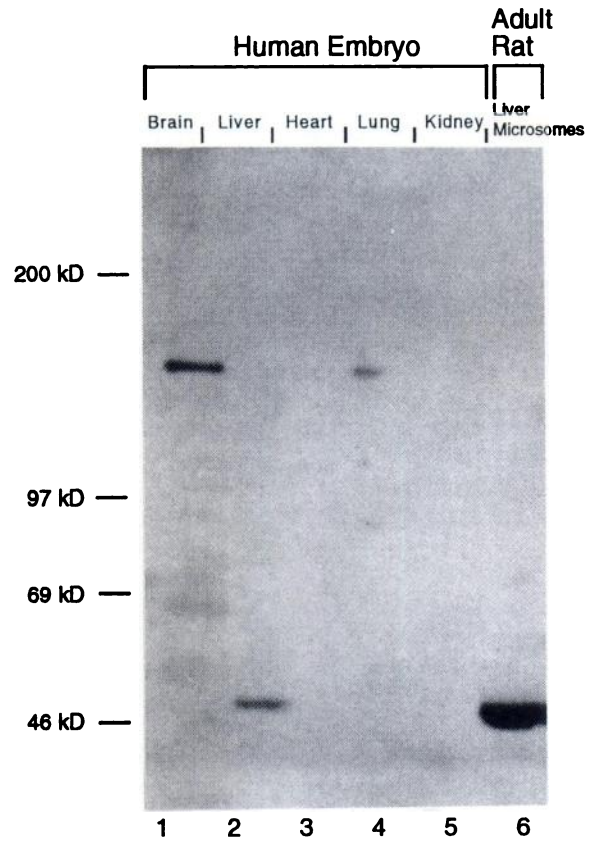

Fig. 2. Immunoblots of 7.5% SDS-polyacrylamide gels. *Lanes 1-5*, human embryonic homogenate 600 × *g* (for 5 min) supernatant fractions (0.04–0.06 mg of protein) from brain (*lane 1*), liver (*lane 2*), heart (*lane 3*), lung (*lane 4*), or kidney (*lane 5*). *Lane 6*, hepatic microsomal fraction prepared from Aroclor-treated adult male rats (0.05 mg of protein).

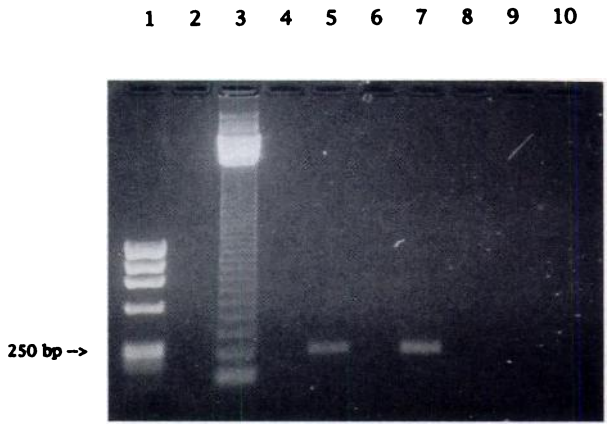

Fig. 3. Ethidium bromide-stained agarose gel showing PCR-amplified cDNA obtained from RT of oligo(dT)-selected mRNA from human embryonic and fetal hepatic tissues. After RT-PCR, reaction mixtures were treated with *Bam*HI, extracted with phenol/chloroform, and precipitated with ethanol. The ethanol-precipitated cDNA was reconstituted in 10 μl of 1× TE buffer (10 mM Tris·HCl, 1 mM EDTA, pH 8.0) and loaded onto a 2% low-melting point agarose gel containing ethidium bromide. *Lanes 1* and *3*, DNA molecular size markers; *lane 5*, cDNA obtained from RT-PCR with human embryonic hepatic mRNA and AMV reverse transcriptase (see Materials and Methods); *lane 7*, cDNA obtained from RT-PCR with human fetal hepatic mRNA and AMV reverse transcriptase; *lane 9*, sample obtained from RT-PCR with human embryonic hepatic mRNA but without AMV reverse transcriptase; *lanes 2*, *4*, *6*, *8*, and *10*, sample blanks. The visible band was 250/259 base pairs in length, as predicted from the nucleotide positions of the PCR primers (with the addition of the *Bam*HI site on the 5′ end of both sense and antisense primers; 240/249 base pairs without the *Bam*HI sites).

Discussion

With respect to prenatal biotransformation and bioactivation of foreign organic chemicals, it is of great importance to determine the extent to which individual, enzymically functional, CYP isoforms are expressed in embryonic tissues during organogenesis. This is because the stage of organogenesis, which occurs during embryonic life rather than during fetal life, is commonly regarded as the stage of development most sensitive to the dysmorphogenic and teratogenic effects of foreign organic chemicals and also because the CYP hemoproteins are so heavily implicated in bioactivation of such chemicals.

The data obtained with (*R*)- and (*S*)-warfarin as probe substrates in this study provide positive evidence for expression of functional CYP3A in human prenatal hepatic tissues during organogenesis in embryos. The use of these two substrates has proven very useful for the identification of multiple distinct isoforms of CYP (Refs. 20, 24, and 31–34 and references cited therein). Selective hydroxylation at the 10-carbon of the *R*-enantiomer is very characteristic of CYP3A isoforms, as has also been recently demonstrated with adult human CYPs (24). However, caution must be exercised in the interpretation of results with only warfarin as a probe for CYP3A isoforms. This has been impressively illustrated in studies (32) in which it was shown that mouse and rat CYP1A1 isoforms differed markedly in terms of their respective substrate specificities for warfarin. It seems clear, as has been emphasized in recent years, that a single probe is insufficient for characterization/identification of CYPs. For these investigations we have utilized multiple probes, each of which yielded data consistent with the concept that enzymically active CYP3A7 is expressed in human embryonic livers. These included three substrate probes [benzyloxyresorufin, (*R*)-warfarin, and (*S*)-warfarin], a highly selective inhibitor (TAO), and an immunoprobe (anti-CYP3A antibody), as well as the cloning and sequencing of RT-PCR-generated cDNA using selective primers. TAO-inhibitable BZROD activity appears to be a particularly selective and highly sensitive probe for functional CYP3A (6, 14, 15, 27). The results of these experiments, however, point out the necessity for caution when tissue fractions that retain cytosolic elements are analyzed, because of our discovery of relatively high BZROD activity associated with the cytosolic fraction. This observation is currently under investigation. In addition, it should be noted that purified CYP3A7 has not been investigated with respect to warfarin and benzyloxyresorufin biotransformation, and it would be premature to infer that the reactions studied are markers for that specific isoform.

In earlier studies with benzyloxyresorufin, an established substrate for isoforms of the CYP3A subfamily (including human CYP3A forms) (14), it was found that, although several human embryonic tissues catalyzed readily measurable and carbon monoxide-inhibitable BZROD activities, the reactions were not inhibited by TAO at concentrations as high as 600 μM and with extended preincubation of enzyme with TAO and cofactors for monooxygenation. Several possible explanations for those observations existed. It was conceivable that CYP3A(s) might not be functionally expressed at detectable levels during the early stage of gestation investigated (50–60 days), i.e., that the BZROD activities observed were catalyzed by other, unidentified, embryonic enzymes that are not effec-

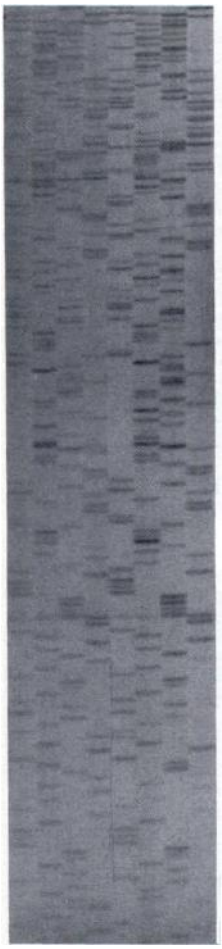

Fig. 4. Partial sequences from antisense strands of the cloned cDNA generated by RT-PCR (see Materials and Methods). *Left four lanes*, partial sequence from one of the 58 colonies whose plasmids each contained an insert with a sequence identical to that present in *CYP3A7* (positions 362–601); *right four lanes*, partial sequence from the colony whose plasmid contained an insert with a sequence identical to that present in *CYP3A5* (positions 446 to 685).

tively inhibited by TAO. Alternatively, it was possible that TAO might not significantly inhibit CYP3A isoforms expressed specifically during the embryonic period or that substances present in the embryonic preparations used as enzyme sources might prevent the inhibitory effect of TAO. It was found that lack of TAO inhibition was due to the presence of components (presumably enzymes) associated with the cytosolic fraction and not separated from the microsomes in those earlier studies. The cytosolic fraction alone was found to be capable of catalyzing the BZROD reactions, exhibiting relatively high activities and accounting for >70% of the activity observed in the 600 × *g* (for 5 min) supernatant fractions used as enzyme sources in earlier studies (14). Because the microsomal fraction accounted for <30% of the total BZROD activity in those preparations and because other human (14) and rodent microsomal CYPs are also capable of catalyzing the reaction, it is now understandable why inhibition by TAO was very weak and at that time regarded as negligible. It is evident that TAO inhibition of functional microsomal CYP3A debenzylase activity was obscured by the dominance of the non-TAO-inhibitable activity associcated with the cytosolic fraction. Identification of the specific components (putative enzymes) responsible for the cytosolic BZROD activity remains a challenging issue for future investigations. Because of observed inhibition by carbon monoxide (data not shown), it might be assumed that the BZROD activity detected in embryonic cytosolic fractions is also CYP dependent, but other hemeproteins present in the fraction might also contribute to catalysis of the debenzylation reaction. Resolution of this aspect also will require further experimentation.

Our results obtained with the tools of molecular biology (RT-PCR, cloning, and sequencing) provided more definitive information pertaining to the identities of the CYP3A isoforms expressed in human embryonic hepatic tissues during organogenesis. The results indicated that CYP3A7 appears to be the strongly predominant CYP3A isoform expressed in these tissues during this early stage of gestation. Evidence for expression of CYP3A5 was detected, but in only one of 59 colonies obtained. CYP3A3 and CYP3A4 were not detected. This pattern was quite similar to that reported earlier for fetal tissues by Wrighton *et al.* (35), who detected CYP3A5 in only one of 10 human fetal livers. In a very recent study, Schuetz *et al.* (36) reported no detection of CYP3A4 mRNA in three of three human fetal livers but reported detection of both CYP3A5 and CYP3A7 in five of five human fetal livers. Interestingly, those authors also reported detectable CYP3A7 mRNA in seven of 13 normal adult human livers, in contrast to the conclusions of others (4, 5, 8, 19) that CYP3A7 is expressed only prenatally except in hepatic tumors or perhaps in hepatic tissues in which other pathological abnormalities might exist. Thus, it appears that a rigorous definition of the pattern of expression of CYP3A isoforms in human embryonic livers, human fetal livers, and even human adult livers will require still further and more definitive investigations.

The results of this investigation affirmatively answer the question of whether functional CYP3A7 is expressed at significant levels in human hepatic tissues during the period of organogenesis, but numerous additional questions remain. One question is whether a homologous CYP3A isoform might be expressed in embryonic hepatic tissues of any other species. At present this seems likely for primates but not for nonprimate species, although only a very few species have been studied. Would the same isoform be expressed during organogenesis in other human tissues? Investigations in this study with immunoblots of embryonic brain, adrenal gland, kidney, lung, and heart now make this seem less probable, but immunoblotting is not an extremely sensitive technique and more careful studies appear to be desirable. Another question pertains to the most important function(s) of CYP3A7. Currently, an evolutionary explanation for expression of high levels of CYP3A4 in adult human liver but undetectable levels in prenatal human liver versus relatively high levels of CYP3A7 in prenatal human liver but reportedly undetectable levels in normal adult human liver remains enigmatic. [One should note that a very recent investigation (36) has called the latter concept into question.] Further investigations will be needed to provide a conclusive answer to this and associated questions. How early in human gestation could functional CYP3A7 be detected? From studies in rats (6, 27), it seems that expression might be expected to coincide with differentation of hepatocytes, although this also will require further study. Whether other CYP isoforms might

be expressed in human tissues during organogenesis is also a question. Evidence exists (14, 15) for the expression of several other isoforms but, as of this writing, rigorous identification of such enzymically functional isoforms in human embryonic tissues has not been reported in the literature. Because the expression of only very low levels of CYPs in embryonic tissues has been shown to be capable of dramatically affecting normal development (37, 38), the answers to these and other related questions assume great importance.

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