Biochemical Pharmacology, Vol. 45, No. 4, pp. 899–907, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

CYTOCHROME P450 ISOFORMS IN HUMAN FETAL TISSUES RELATED TO PHENOBARBITAL-INDUCIBLE FORMS IN THE MOUSE

JUKKA MÄENPÄÄ,* ANDERS RANE,† HANNU RAUNIO, PAAVO HONKAKOSKI‡ and OLAVI PELKONEN

Department of Pharmacology and Toxicology, University of Oulu, SF-90220 Oulu;
‡Department of Pharmacology and Toxicology, University of Kuopio, SF-90721 Kuopio, Finland;
and †Department of Clinical Pharmacology, Akademiska Hospital, University of Uppsala, S-751 85 Uppsala, Sweden

(*Received* 9 *June* 1992; *accepted* 23 *November* 1992)

Abstract—Four polyclonal antibodies raised against purified mouse liver cytochrome P450s representing *Cyp1a*, *Cyp2a*, *Cyp2b* and *Cyp2c* subfamilies were used to detect their related forms in human adult and fetal tissues. In immunoblot analysis, anti-Cyp2c antibody detected two to three proteins in adult livers and one to three proteins in 70% of the 18 fetal livers studied. Anti-Cyp2a-5 antibody recognized a 50-kDa protein in 50% of the fetal adrenals. Anti-Cyp1a-2 antibody reacted with a single protein (55 kDa) in adult liver. The anti-Cyp2b-10 antibody did not detect proteins in any of the tissues. No proteins were detected in fetal kidneys. There was no coumarin 7-hydroxylase activity (COH) in fetal liver or adrenals. The 7-ethoxycoumarin *O*-deethylase (ECOD) activities were slightly higher in fetal adrenals (mean 6.1 pmol/mg protein/min) vs livers. The fetal adrenal ECOD activity was not inhibited by the anti-Cyp2a-5 antibody. Aryl hydrocarbon hydroxylase (AHH) activities in fetal livers were about 5% of those in adult livers. AHH activity in fetal liver was not inhibited by the anti-Cyp2c antibody. Testosterone 6β-hydroxylase activity was much lower in fetal liver than in adult liver (about 20 and 1700 pmol/mg protein/min, respectively). No immunoinhibition occurred in fetal adrenal progesterone hydroxylation, hepatic benzphetamine N-demethylation and hepatic ethylmorphine N-demethylation. These data suggest that members of the P450 subfamilies 1A, 2A and 2B are expressed at a very low level in fetal liver, and that fetal liver may contain members of the 2C subfamily.

The cytochrome P450 enzymes are the terminal components of the microsomal monooxygenase system catalysing the oxidation of both exogenous and endogenous compounds [1]. Human liver microsomes contain multiple P450 isoforms which have been classified into families and subfamilies based on their DNA sequence similarity [2].§ Xenobiotic-metabolizing P450s in families CYP1–4 have been detected in several tissues in the adult human [1, 2]. In contrast, only a few cytochrome P450 isoforms and restricted substrate specificities have been demonstated in human fetal liver [3–8].

Four different cytochrome P450s representing the *Cyp1a*, *Cyp2a*, *Cyp2b* and *Cyp2c* subfamilies have been purified from phenobarbital-induced mouse liver [9–10]. In a previous study [11] we showed that all the antibodies (except anti-Cyp2b-10) raised against these isoforms detected P450s in adult human liver microsomes. The antibodies also inhibited several model substrate reactions, agreeing with other reports in the literature where comparisons could be made.

The possibility of cross-reactions with non-related P450 isoforms exists when heterologous antibodies are used. However, Forrester *et al.* [12] showed recently that surprisingly few cross-reactions occurred among human isoforms in families 1 through 4 when polyclonal anti-rat P450 antibodies were used. A small number of human isoforms have been purified to date [13], and even fewer well-characterized antibodies against human isoforms are available. The aim of this work was to investigate whether P450s recognizable by the anti-mouse P450 antibodies exist in human fetal and adult tissues.

MATERIALS AND METHODS

Chemicals. Reagents used for protein immunoblotting were purchased from Bio-Rad (Richmond, CA, U.S.A.), E. Merck (Darmstadt, F.R.G.), Sigma (St Louis, MO, U.S.A.) or Zymed Laboratories Inc. (San Francisco, CA, U.S.A.). Coumarin, ethoxycoumarin, 7-hydroxycoumarin, benzo(*a*)-pyrene and testosterone were from Sigma. Benzphetamine hydrochloride was from the Upjohn Co. (Kalamazoo, MI, U.S.A.) and ethylmorphine hydrochloride from the University of Uppsala Hospital Pharmacy. [4-^{14}C]Testosterone (57 mCi/mmol) was from Amersham (Amersham, U.K.). All other chemicals were at least of analytical grade.

Human fetal and adult tissue specimens and microsomal preparation. Tissue specimens from 20 fetuses between 13 and 23 weeks of gestation were

* Corresponding author. Tel (358) 81 537 52 52; FAX (358) 81 33 06 87.

§ The latest P450 nomenclature [2] will be used in this article. The mouse *Cyp2a-5* gene encodes for Cyp2a-5 protein which has high coumarin 7-hydroxylase activity. The *Cyp2a-4* gene product is testosterone 15α-hydroxylase. The *CYP2A6* gene product catalyses coumarin 7-hydroxylase in human liver.

obtained at abortions performed for socio-medical reasons. The abortions were performed by prostaglandin $PGF_{2\alpha}$ infusion (6.5 hr). The tissues (liver, adrenals, kidneys, brain) were excised within 60 min of the abortion. Human adult liver specimens were obtained at surgery (livers 1–6). Two samples of adult liver were prepared from organ donors who had died accidentally (livers 15 and 16). All the samples were frozen at −80° until assay. The project was approved by the Ethics Committees of Uppsala and Oulu Universities. The microsomes from human fetal and adult tissues were isolated by ultracentrifugation as described [14]. The protein content was measured according to Lowry *et al.* [15].

Preparation of antibodies. The P450 isoforms (trivial names P450PbI, P450PbII, P450PbIII and P450Coh) were purified from phenobarbital or pyrazole-treated mouse liver as described [9, 10]. N-terminal amino acid sequencing (at least 20 residues) showed that P450PbI is 100% similar to Cyp2b-10 [16], P450PbII is 100% similar to Cyp1a-2 and P450PbIII is 89% similar to CYP2C7 [10]. The fourth isoform P450Coh has been shown definitively to be the product of the *Cyp2a-5* gene [17]. The isoforms have therefore been redesignated Cyp1a-2 (P450PbII), Cyp2a-5 (P450Coh), Cyp2b-10 (P450PbI) and Cyp2c (P450PbIII). A gene designation cannot be given for *Cyp2c* since the complete nucleotide sequence is not known for the respective gene. Antisera against these P450 isoforms were raised in rabbits and the immunoglobulin G fractions were purified by $(NH_4)_2SO_4$ precipitation.

Protein electrophoresis and immunoblot techniques. SDS–PAGE was performed according to Laemmli [18]. Polyacrylamide (10%) in 0.75 mm of 1.0 mm gels was used in a Mini-protean II dual slab cell equipment (Bio-Rad). Microsomal protein (10–30 μg) was applied to each well. The proteins were transferred to nitrocellulose membranes in 1 hr according to Towbin *et al.* [19]. The membranes were placed first in plastic bags with phosphate-buffered saline, pH 7.5 containing 0.05% Tween 20 and 1% or 5% non-fat milk powder (blocking solution). One hour blocking (37°) was found to be sufficient, but somewhat better results were obtained by using longer blocking times of up to 18 hr. The membranes were then incubated with the primary antibody for 1–1.5 hr. The secondary antibody was a goat anti-rabbit–alkaline phosphatase conjugated antibody. Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were used as color development reagents. Between each step the filters were washed extensively with phosphate-buffered saline containing 0.05% Tween 20.

Enzyme assays. Coumarin 7-hydroxylase (COH*) and ethoxycoumarin *O*-deethylase (ECOD) activities were measured according to Aitio [20]. COH and ECOD activities were measured at a saturating (0.1 mM) substrate concentration and the incubation time was 20 or 30 min. Testosterone 6β-hydroxylase activity was measured according to Waxman *et al.* [21] as described in detail earlier [22]. Aryl hydrocarbon hydroxylase (AHH), benzphetamine *N*-demethylase and ethylmorphine *N*-demethylase activities were measured as described [23–25]. Progesterone 16α-/17α-hydroxylation and 21-hydroxylation of progesterone to desoxycorticosterone and 11-deoxycortisol as a secondary metabolite were measured according to Taylor *et al.* [26] as modified by Rane and Ask [27].

Immunoinhibition studies. The antibody was added to the incubation mixture at room temperature 10 min before the initiation of 2 min preincubation at 37°. The ratio of antibody/microsomal protein (1:1–5:1) used was based on earlier reports [11, 28]. Preimmune immunoglobulin G was used as a control in every assay.

RNA blots. Total RNA from adult and fetal liver was prepared by the cesium chloride centrifugation method [29]. Aliquots (20 μg) of total RNA were separated on a 1% agarose gel followed by transfer to a nylon membrane (Hybond-N; Amersham, U.K.). The integrity of the RNA was checked by ethidium bromide staining. The RNA was fixed by baking at 80° and was then hybridized with a ^{32}P-labeled human *CYP2A6* probe (Salonpää *et al.*, unpublished). The filters were washed at 55° in 0.1 × SSC/0.1% SDS (3 × 20 min). The filter was exposed to autoradiography film for 1 week at −70°.

Table 1. Clinical data on the fetal samples

Fetus	Gestational age (weeks)	Smoking status
1	23	Not known
8	22	−
12	19	+
15	14–15	Not known
16	15	−
17	16	−
22	16–17	−
24	15	Not known
25	15	Not known
26	15	−
27	15	−
28	16	+
29	16	+
30	13	−
31	16	Not known
34	17–18	+
35	14	−
36	16–17	+
37	18	−
44	14	+

Non-smoking mothers have been marked with − and smokers with +. None of the mothers was known to have used drugs regularly. The mother of fetus 34 had a history of alcohol usage during pregnancy.

RESULTS

Clinical data

The gestational age of the fetuses and the smoking status of the mothers are listed in Table 1. Table 2

* Abbreviations: COH, coumarin 7-hydroxylase; ECOD, 7-ethoxycoumarin *O*-deethylase; AHH, aryl hydrocarbon hydroxylase; 15αOH, testosterone 15α-hydroxylase; SSC, standard saline citrate; SDS, sodium dodecyl sulfate.

Table 2. Clinical data on the adult samples

Adult	Age (years)	Sex	Smoking status	Drugs
1	66	Female	Not known	—
2	71	Male	+	Digoxin, Metoprolol, Bendroflumethiazide
4	55	Female	Not known	Dixyrazine, Buprenorfine, Flunitrazepam, Nitrazepam
5	64	Male	+	—
6	29	Male	−	Penicillamine
15	20	Male	Not known	Not known
16	47	Female	Not known	Not known

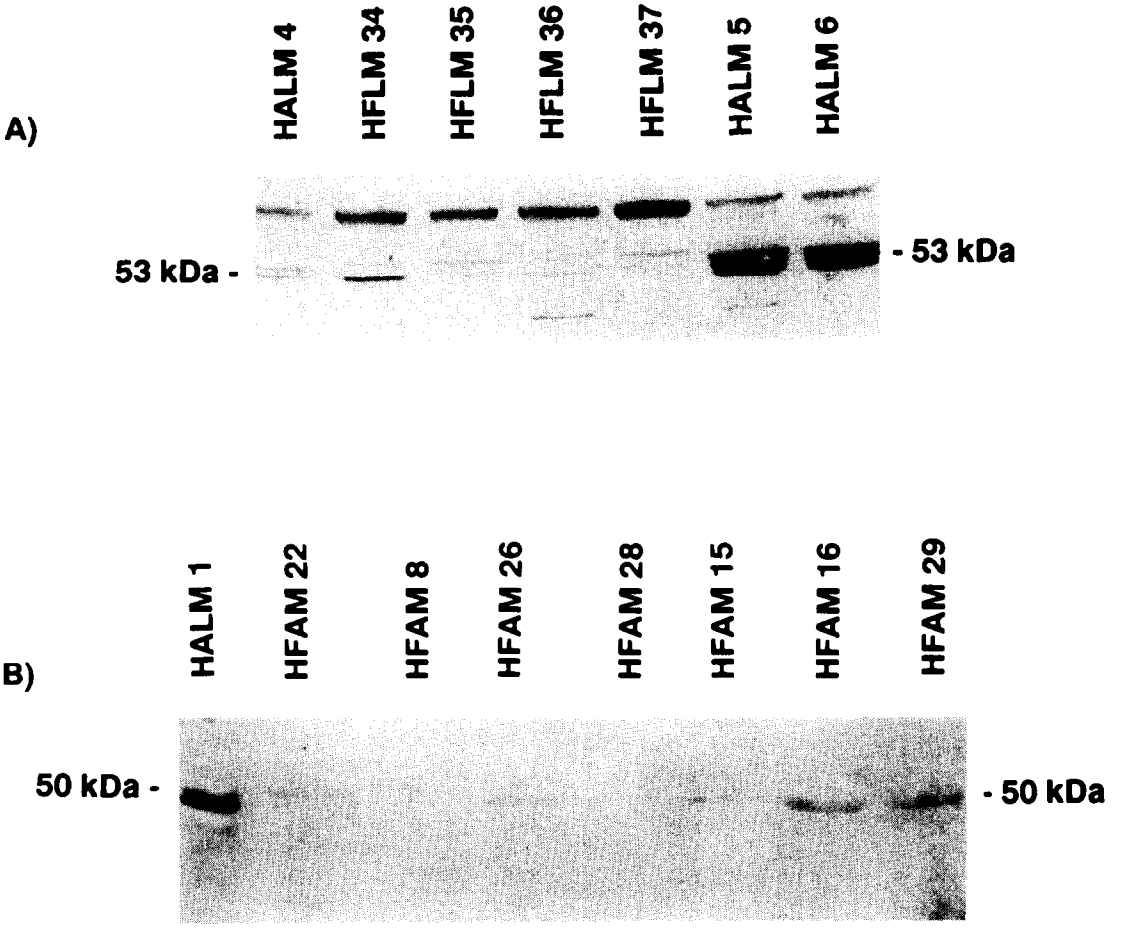

Fig. 1. (A) Protein immunoblot with anti-Cyp2c antibody of human adult (HALM) and fetal liver (HFLM). (B) Protein immunoblots with anti-Cyp2a-5 antibody of human adult liver (HALM) and fetal adrenal microsomes (HFAM). Ten micrograms (adult liver), 20 μg (fetal adrenals) and 30 μg (fetal liver) of microsomal protein were applied in the lanes.

shows the age, sex, smoking status and drug usage of the adult donors.

Immunoblot analysis

Several fetal and adult tissues were screened with the antibodies in protein immunoblots (Fig. 1, Table 3). Figure 1A shows an immunoblot of three adult and four fetal livers with the anti-Cyp2c antibody. The antibody detected two or three bands (51, 53 and 54.5 kDa) in the six adult livers studied (Table 3). In a total of 18 fetal liver samples studied, a single band (54.5 or 55 kDa) was detected in seven, two bands in four (53 and 55 kDa) (Fig. 1A) and three bands in one liver (51, 53 and 54.5 kDa). The

Table 3. Immunoblot analysis of fetal and adult tissue microsomes

Tissue	Antibody 1a-2	2a-5	2b-10	2c
Adult				
Liver	3/3	3/3	0/1	6/6
Fetus				
Liver	0/10	0/12	0/5	12/18
Adrenal	0/7	8/15	0/7	2/7
Brain	0/6	0/6	0/6	2/6
Kidney	0/5	0/7	0/5	0/3

The numbers denote positive immunoblots/samples studied.

antibody did not detect any bands in the P450 molecular mass area in six fetal livers (Table 3). Two unspecific bands (>70 kDa and <40 kDa) were observed in most adult and fetal livers by the anti-Cyp2c antibody (Fig. 1A). A very faint 51-kDa band was seen in two out of six fetal brains (Table 3).

Figure 1B shows an immunoblot of one adult liver and seven fetal adrenals with the anti-Cyp2a-5 antibody. The antibody detected the 49-kDa CYP2A6 protein in all adult livers. Also a weak 50-kDa band was detected in most adult livers. No bands could be seen in any of the 12 fetal liver samples studied, whereas a 50-kDa band was detected in eight out of 15 fetal adrenals (Fig. 1B, Table 3).

No proteins cross-reacted in any of the fetal tissues with the anti-Cyp1a-2 antibody, which detects a 55-kDa protein in adult liver [11]. Anti-Cyp2b-10 did not detect any proteins in either adult or fetal tissues (blots not shown). In addition to the data presented above, no bands were detected by any of the antibodies in the fetal kidney samples (Table 3).

Activities of marker enzymes

ECOD activities in fetal liver microsomes were fairly low (maximum 3.3 pmol/mg protein/min) when compared with adult liver activities (mean value 160 pmol/mg protein/min) (Table 4). AHH activity was studied in seven fetal liver samples. The mean value was about 6 pmol/mg protein/min with one sample showing a fairly high activity of 21 pmol/mg protein/min. The fetal liver AHH activities were about 5–6% of those in adult livers. Benzphetamine *N*-demethylase activity was studied in four fetal livers. The activities ranged from 420 to 1100 pmol/mg protein/min which is about 45% of the adult liver activity (Table 4). Testosterone 6β-hydroxylase activity was detectable in every fetal liver although it was only about 1% of the adult liver activity (Table

Table 4. Enzyme activities and the presence of anti-Cyp2c antibody-detectable protein in adult and fetal livers

	ECOD	AHH	Testosterone 6β-hydroxylase (pmol/mg protein/min)	Benzphetamine *N*-demethylase	Cyp2c
Adult liver					
1	190	120	1900	1400	+
2	120	74	1500	2000	+
Mean	160	97	1700	1700	
Fetal liver					
1	1.9	ND	ND	ND	+
8	1.2	ND	9.6	ND	+
12	3.3	ND	ND	ND	+
15	<1.0	ND	7.4	ND	−
16	<1.0	ND	ND	ND	−
17	<1.0	1.6	ND	ND	+
22	<1.0	21	47	580	−
24	<1.0	ND	ND	ND	−
25	ND	3.1	ND	ND	+
26	1.4	ND	39	840	−
27	<1.0	2.2	ND	420	+
28	<1.0	4.2	8.8	ND	+
29	<1.0	7.5	13	510	−
30	<1.0	4.6	7.8	ND	+
34	ND	ND	58	1100	+
36	ND	ND	35	840	+
37	ND	ND	17	1100	+
Mean	<1.0	6.2	24	770	
SD	0.92	6.6	19	280	

ND, not determined due to insufficient amount of material.
<1.0 denotes values less than 1 pmol/mg protein/min.
+, positive staining with anti-Cyp2c antibody.

Table 5. ECOD activity and the presence of the proteins detected by anti-Cyp2a-5 and anti-Cyp2c antibodies in fetal adrenals

Adrenal	ECOD	Cyp2a-5	Cyp2c
8	<1.0	ND	ND
15	11	+	ND
16	27	+	ND
17	9.0	−	ND
22	2.6	+	+
24	ND	−	−
25	3.9	−	−
26	6.7	+	−
27	<1.0	−	−
28	<1.0	−	−
29	8.2	+	+
30	<1.0	+	ND
31	<1.0	−	ND
34	3.7	+	ND
36	12.6	+	ND
37	ND	−	ND
Mean	6.1		
SD	7.4		

ECOD activity is given in pmol/mg protein/min.
<1.0 denotes values less than 1 pmol/mg protein/min.
ND, not determined.
+, positive staining with anti-Cyp2a-5 and anti-Cyp2c antibodies.

4). No COH activity was detected in the 12 fetal livers studied. Adult liver samples (1, 2, 15 and 16) had COH activities ranging from 190 to 710 pmol/mg protein/min.

Anti-Cyp2a-5 antibody detected a protein in about 50% of the fetal adrenal samples studied. Therefore, the activities of COH and ECOD, both known to be mediated by the human CYP2A6 [28, 30], were measured in fetal adrenal microsomes. No COH activity was detected in any of the 13 adrenals studied. In contrast, ECOD activity was present at fairly high levels in most of the adrenal samples (Table 5). The ECOD activities did not correlate well with the intensities of the bands detected by the anti-Cyp2a-5 antibody.

Table 7. The effect of the anti-Cyp2c antibody on AHH, benzphetamine *N*-demethylase and ethylmorphine *N*-demethylase activities in human adult and fetal liver microsomes

	AHH	Benzphetamine	Ethylmorphine
	% of control activity (± SD)		
Adult liver	58 ± 23*	99 ± 21†	ND
Fetal liver	119 ± 32†	91 ± 22‡	101 ± 32‡

* N = 8. The data is partly from [11].
† N = 3.
‡ N = 4.
ND, not determined.

Immunoinhibition studies

Immunoinhibition studies were performed to evaluate the contribution of individual P450s in some of the activities observed. Anti-Cyp2a-5 antibody did not inhibit ECOD activity in any of the five fetal adrenal samples studied. Consistent with previous studies [11], anti-Cyp2a-5 inhibited ECOD activity in adult livers (Table 6). Progesterone is known to be hydroxylated in the human fetal adrenals [31]. The anti-Cyp2a-5 antibody had no clear effect on 16α-, 17α- and 21-hydroxylation of progesterone in the four adrenal samples tested (Table 6).

Although anti-Cyp2c has been shown to inhibit AHH activity by up to 72% in adult livers [11], it did not inhibit AHH activity at all in fetal livers (Table 7). Anti-Cyp2c also did not inhibit significantly benzphetamine *N*-demethylase activity in fetal and adult livers (Table 7). Ethylmorphine *N*-demethylase activity (range 167–1210 pmol/mg protein/min) was not inhibited considerably by the anti-Cyp2c antibody in four fetal liver specimens (Table 7).

RNA blot analysis

RNA blot analysis was performed with two adult and fetal livers using a full-length human *CYP2A6*

Table 6. The effect of anti-Cyp2a-5 antibody on ECOD activity in human adult liver and human fetal adrenal specimens and on progesterone metabolism in fetal adrenals

	ECOD	DOCOL	16αOH	17αOH	DOC
	% of control activity (± SD)				
Adult liver	58 ± 18*	ND	ND	ND	ND
Fetal adrenals	108 ± 45†	103 ± 11‡	118 ± 28‡	115 ± 28‡	109 ± 22‡

* N = 9. The data is partly from [11].
† N = 5.
‡ N = 4.
ND, not determined.
DOCOL and DOC, 21-hydroxylation of progesterone to 11-desoxycortisol and to 11-desoxycorticosterone, respectively. 16αOH, 16α-hydroxylation of progesterone. 17αOH, 17α-hydroxylation of progesterone.

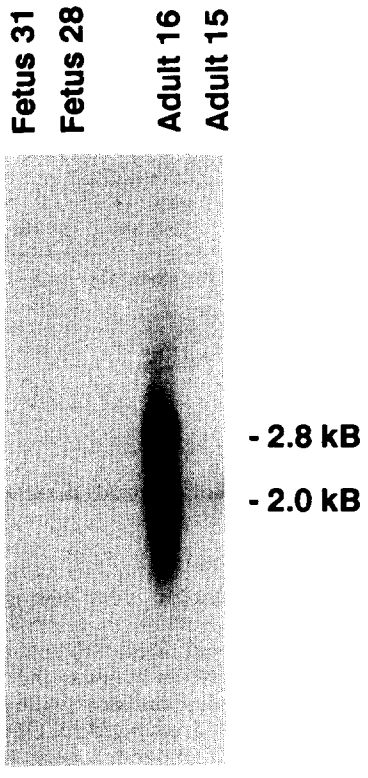

Fig. 2. RNA blot analysis of RNA from selected human fetal and adult livers. Total RNA (20 μg) was electrophoresed in an agarose gel and transferred onto nylon membrane, which was hybridized with a ^{32}P-labeled CYP2A6 cDNA. kB, kilobase.

cDNA as the probe. A substantial amount of 2.0- and 2.8-kb mRNA species was detected by the probe in adult liver 16 (Fig. 2). The transcript sizes are in agreement with an earlier report of Yamano *et al.* [30]. Upon longer exposure a 2.0-kb mRNA could also be seen in adult liver 15, but no mRNA signals were detected in the fetal liver samples. This result correlated well with the amount of anti-Cyp2a-5-detectable CYP2A6 protein and COH activities in adult livers 15 and 16.

DISCUSSION

In this work we have attempted to ascribe some P450-mediated activities in human fetal tissues to specific P450 isoforms using a panel of anti-mouse P450 antibodies. The main finding of this study is that P450 isoforms which exist in several adult tissues appear to be absent or expressed at a very low level in the corresponding human fetal tissues.

ECOD and AHH activities are detectable in human fetal liver [6, 32, 33] and ECOD activity is also found in fetal adrenals [8, 32, 34]. COH is high in adult liver [11, 35] and the activity is almost completely inhibited by an anti-Cyp2a-5 antibody. Cyp2a-4 and Cyp2a-5 in mouse liver differ in only 11 out of 494 amino acid residues [17]. Cyp2a-4 catalyses the 15α-hydroxylation of testosterone. This activity in human adult liver microsomes is only 5–10% of mouse testosterone 15α-hydroxylase (15αOH) activity and it is not inhibited by anti-Cyp2a-5 antibody like mouse 15αOH [22]. There is no 15αOH activity in human fetal liver [36]. The present results do not reveal any COH activity in fetal liver or adrenals, even though some fairly mature fetuses (up to 23 weeks) were studied. In immunoblots of fetal liver microsomes no immunoreactive protein was detected with the anti-Cyp2a-5 antibody. In RNA blots, no hybridizable mRNA species were detected by the CYP2A6 cDNA probe in fetal liver. Therefore, the period in human ontogenic development in which CYP2A6 begins to be expressed remains uncertain. It probably occurs soon after birth, since Crespi *et al.* [37] have found an immunoreactive protein with a P4502A antibody and also measurable levels of COH activity in human infant liver microsomes, although the activities were lower than in adult liver [11].

In 50% of the fetal adrenals studied here, a faint 50-kDa protein was detected with the anti-Cyp2a-5 antibody. A similar size protein is often detected in adult liver samples in addition to the 49-kDa CYP2A6 [11, 28, 30, 38]. No COH activity was present in the fetal adrenal samples. The adrenal ECOD activity was not inhibited by the anti-Cyp2a-5 antibody, which has been shown to inhibit adult liver ECOD activity in the present and previous works [11, 38]. The antibody had also no effect on progesterone metabolism in the adrenals. Consequently, the identity of the protein detected by this antibody in fetal adrenals in unclear. Anti-CYP3A antiserum has also been shown to lack inhibitory activity towards fetal adrenal ECOD activity [32], suggesting that the CYP3A subfamily may also not be involved in this activity.

The ECOD activities in the fetal livers were very low and also no proteins were detected by the anti-Cyp2a-5 antibody in immunoblots. This suggests that the CYP2A6 is not responsible for the low ECOD activity in fetal liver. The ability of anti-CYP3A antiserum to inhibit ECOD activity suggests that members in the CYP3A subfamily are involved in the constitutive ECOD activity in fetal liver [8, 32].

CYP2B genes are expressed in the human liver [39, 40], but no proteins have yet been purified in this subfamily. Human liver microsomal proteins presumed to be CYP2B isoforms have been detected with anti-rat CYP2B antibodies in immunoblots by others [12, 40]. The anti-Cyp2b-10 antibody did not detect any clear protein bands in the fetal liver or other tissues studied here. This is also the case in the adult liver [11]. We have also failed to detect any immunoreactive bands with monoclonal anti-rat CYP2B antibody (Mab 2-66-3) in adult liver microsomes (Raunio *et al.*, unpublished). This is in agreement with Wrighton *et al.*, [41], who did not detect any cross-reacting proteins in over 50 human liver samples with a panel of 12 monoclonal antibodies against rat CYP2B. Since the positive findings with anti-rat CYP2B antibodies can be due to cross-reactions with other human isoforms, the issue of the existence of constitutive CYP2B proteins in human liver appears unresolved at the moment.

The *CYP1A* subfamily consists of two different genes. Human adult liver contains a gene encoding CYP1A2, whereas CYP1A1 is found in many extrahepatic tissues [42]. A monoclonal antibody against rat CYP1A1 was reported recently to recognize a protein in immunohistochemical analysis in human adult and fetal liver [43]. Although the anti-Cyp1a-2 antibody detects CYP1A2 in adult liver [11], microsomes from human fetal tissues (liver, adrenals, kidneys and brain) did not react with this antibody. A monoclonal antibody anti-rat CYP1A (Mab 1-7-1) raised against rat CYP1A [44] also failed to detect any protein in fetal liver in immunoblots [8]. This suggests that CYP1A isoforms are expressed at very low levels in human fetal liver.

To date, five members of the human *CYP2C* subfamily have been characterized [45]. In the present work, adult liver microsomes were found to contain two to three different proteins immunoreactive with the anti-Cyp2c antibody. Two groups [12, 46] have reported that an anti-rat CYP2C antiserum detects three proteins in adult liver microsomes. Doecke *et al.* [47] made the same observation with anti-rabbit CYP2C3 antiserum. Four groups have used three independent antibody preparations raised against CYP2C9 and only one or two proteins were reported to cross-react in human adult liver [6, 45, 48, 49]. Likewise, Leo *et al.* [50] reported the detection of one protein in human liver microsomes by an anti-human CYP2C8 antibody. It thus appears that antibodies made against members of the rodent CYP2C subfamily cross-react with more than two proteins in human adult liver, which may be due to their broader epitope detection capacity compared with antibodies made against human proteins.

Until this paper there were no reports on the presence of putative CYP2C proteins in fetal liver. Cresteil *et al.* [6] and Pons *et al.* [46] reported that anti-human CYP2C9 or anti-rat CYP2C11 antibodies did not cross-react with any proteins in several fetal livers. There is no mephenytoin hydroxylase activity (associated with CYP2C subfamily) [51] or CYP2C9-related mRNA in fetal liver [52]. In the present report the anti-Cyp2c antibody cross-reacted with one to three proteins in 70% of the fetal livers studied. The detected proteins correspond in size with the three proteins detected in adult liver samples. These proteins may represent members of the CYP2C subfamily, but more specific and sensitive methods need to be applied before an unequivocal conclusion can be made. The faint staining in immunoblots of fetal brain samples by anti-Cyp2c antibody cannot at present be ascribed to P450 proteins, although the human brain appears to contain fairly high levels of total P450 [53].

A purified mouse Cyp2c protein has ethylmorphine N-demethylase activity [54] and benzphetamine is N-demethylated by human CYP2C8 [55]. Nevertheless, in the present work these activities were not significantly inhibited by the anti-Cyp2c antibody in fetal liver. In a previous study [11], AHH activity was inhibited 29–72% by the anti-Cyp2c antibody in adult liver microsomes. Anti-Cyp2c antibody did not, however, inhibit the low constitutive AHH activity in fetal liver microsomes. This suggests that the anti-Cyp2c reactive P450s do not metabolize benzo(*a*)pyrene in fetal liver. Cresteil *et al.* [6] showed that AHH activity correlates with CYP2C9 concentration in adult livers. AHH is inhibited by anti-CYP3A7 antibody in fetal liver [32]. Hence, it is conceivable that different isoforms are catalysing benzo(*a*)pyrene in human adult and fetal liver microsomes.

Acknowledgements—We thank Danuta Thisner and Birgitta Ask for their contribution to this work. This study was sponsored by the Swedish Medical Research Council (04X-04496), the Expressen Prenatal Research Foundation and the Academy of Finland (Medical Research Council Contract No. 1051029).

REFERENCES

1. Gonzalez FJ, The molecular biology of cytochrome P450s. *Pharmacol Rev* **40**: 243–288, 1989.
2. Nebert DW, Nelson DR, Coon MJ, Estabrook RW, Feyereisen R, Fujii-Kuriyama Y, Gonzalez FJ, Guengerich FP, Gunsalus IC, Johnson EF, Loper JC, Sato R, Waterman MR and Waxman DJ, The P450 superfamily: update on new sequences, gene mapping, and recommended nomenclature. *DNA Cell Biol* **10**: 1–14, 1991.
3. Yaffe SJ, Rane A, Sjöqvist F, Boreus L-O and Orrenius S, The presence of monooxygenase system in human fetal liver microsomes. *Life Sci* **9**: 1189–1200, 1970.
4. Pelkonen O, Biotransformation of xenobiotics in the fetus. *Pharmacol Ther* **10**: 261–281, 1980.
5. Kitada M, Kamataki T, Itahashi K, Rikihisa T, Kato R and Kanakubo Y, Purification and properties of cytochrome P-450 from homogenates of human fetal livers. *Arch Biochem Biophys* **241**: 275–280, 1985.
6. Cresteil T, Beaune P, Kremers P, Celier C, Guengerich P and Leroux J-P, Immunoquantification of epoxide hydrolase and cytochrome P-450 isozymes in fetal and adult human liver microsomes. *Eur J Biochem* **151**: 345–350, 1985.
7. Komori M, Nishio K, Fujitani T, Ohi H, Kitada M, Mima S, Itahashi K and Kamataki T, Isolation of a new human fetal liver cytochrome P450 cDNA clone: evidence for expression of a limited number of forms of cytochrome P450 in human fetal livers. *Arch Biochem Biophys* **272**: 219–225, 1989.
8. Ladona MG, Park SS, Gelboin HV, Hammar L and Rane A, Monoclonal antibody directed detection of cytochrome P-450 (PCN) in human fetal liver. *Biochem Pharmacol* **37**: 4735–4741, 1988.
9. Juvonen R, Shkumatov V and Lang M, Purification and characterization of a liver microsomal cytochrome P-450 isozyme with a high affinity and metabolic capacity for coumarin from pyrazole-treated D2 mice. *Eur J Biochem* **171**: 205–211, 1988.
10. Honkakoski P and Lang MA, Mouse liver PB-inducible P450 system. Purification, characterization and differential inducibility of four cytochrome P450 isozymes from D2 mouse. *Arch Biochem Biophys* **273**: 42–57, 1989.
11. Raunio H, Valtonen J, Honkakoski P, Lang MA, Ståhlberg M, Kairaluoma MA, Rautio A, Pasanen M and Pelkonen O, Immunochemical detection of human liver cytochrome P450 forms related to phenobarbital-inducible forms in the mouse. *Biochem Pharmacol* **40**: 2503–2509, 1990.
12. Forrester LM, Hendersson CJ, Glancey MJ, Back DJ, Park BK, Ball SE, Kitteringham NR, McLaren AW, Miles JS, Skett P and Wolf CR, Relative expression of cytochrome P450 isoenzymes in human liver and

association with the metabolism of drugs and xenobiotics. *Biochem J* **281**: 359–368, 1992.
13. Wrighton SA and Stevens JC, The human hepatic cytochromes P450 involved in drug metabolism. *Crit Rev Toxicol* **22**: 1–21, 1992.
14. Rane A, Gawronska-Szklarsz B and Svensson J-O, The natural (−)- and unnatural (+)-enantiomers of morphine. Comparative metabolism and effect of morphine and phenobarbital treatment. *J Pharmacol Exp Ther* **234**: 761, 1985.
15. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
16. Noshiro M, Lakso M, Kawajiri K and Negishi M, Rip locus: regulation of female-specific isozyme (1-P-$450_{16\alpha}$) of testosterone 16α-hydroxylase in mouse liver, chromosomal localization, and cloning of P-450 cDNA. *Biochemistry* **27**: 6434–6443, 1988.
17. Negishi M, Lindberg R, Burkhart B, Ichikawa T, Honkakoski P and Lang M, Mouse steroid 15α-hydroxylase gene family: identification of type II P-$450_{15\alpha}$ as coumarin 7-hydroxylase. *Biochemistry* **28**: 4169–4172, 1989.
18. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
19. Towbin H, Staehelin T and Gordon J, Electrophoretic transfer of proteins from polyacrylic gels to nitrocellulose: procedure and some applications. *Proc Natl Acad Sci USA* **76**: 4350–4354, 1979.
20. Aitio A, A simple and sensitive assay of 7-ethoxycoumarin deethylation. *Anal Biochem* **85**: 488–491, 1978.
21. Waxman DJ, Ko A and Walsh C, Regioselectivity and stereoselectivity of androgen hydroxylations catalyzed by cytochrome P-450 isozymes purified from phenobarbital-induced rat liver. *J Biol Chem* **258**: 11937–11947, 1983.
22. Mäenpää J, Syngelmä T, Honkakoski P, Lang MA and Pelkonen O, Comparative studies on coumarin and testosterone metabolism in mouse and human livers: differential inhibitions by the anti-P450Coh antibody and metyrapone. *Biochem Pharmacol* **42**: 1229–1235, 1991.
23. Nebert DW and Gelboin HV, Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. I. Assay and properties of induced enzyme. *J Biol Chem* **243**: 6242–6249, 1968.
24. Cochin J and Axelrod J, Biochemical and pharmacological changes in the rat following chronic administration of morphine, nalorphine and normorphine. *J Pharmacol Exp Ther* **125**: 105–110, 1959.
25. Ladona MG, Spalding DJM, Ekman L, Lindström B and Rane A, Human fetal and adult liver metabolism of ethylmorphine: relation to immunodetected cytochrome P-450 PCN and interactions with important fetal corticosteroids. *Biochem Pharmacol* **38**: 3147–3155, 1989.
26. Taylor RB, Kendle KE and Reid RG, Chromatography of progesterone and its major metabolites in rat plasma using microbore high-performance liquid chromatography columns with conventional injection and detection systems. *Chromatography* **385**: 383–392, 1987.
27. Rane A and Ask B, A conspicuous down-regulating effect of morphine on essential steroid hydroxylation reactions and on certain drug N-demethylations. *J Steroid Biochem Mol Biol* **41**: 91–98, 1992.
28. Raunio H, Syngelmä T, Pasanen M, Juvonen R, Honkakoski P, Kairaluoma MA, Sotaniemi E, Lang MA and Pelkonen O, Immunochemical and catalytical studies on hepatic coumarin 7-hydroxylase in man, rat, and mouse. *Biochem Pharmacol* **37**: 3889–3895, 1988.
29. Chirgwin JJ, Przbyla AE, MacDonald RJ and Rutter WJ, Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**: 5294–5299, 1979.
30. Yamano S, Tatsuno J and Gonzalez FJ, The CYP2A3 gene product catalyzes coumarin 7-hydroxylation in human liver microsomes. *Biochemistry* **29**: 1322–1329, 1990.
31. Rane A, Henningsson S, Ask B and Ladona MG, Comparison of human fetal hepatic and adrenal cytochrome P450 activities with some major gestational steroids and ethylmorphine as substrates. *J Steroid Biochem Mol Biol* **43**: 335–341, 1992.
32. Kitada M, Kamataki T, Itahashi K, Rikihisa T, Kato R and Kanakubo Y, Immunochemical examinations of cytochrome P-450 in various tissues of human fetuses using antibodies to human fetal cytochrome P-450, P-450 HFLa. *Biochem Biophys Res Commun* **131**: 1154–1159, 1985.
33. Pasanen M, Pelkonen O, Kauppila A, Park SS, Friedman FK and Gelboin HV, Characterization of human fetal hepatic cytochrome P-450-associated 7-ethoxyresorufin *O*-deethylase and aryl hydrocarbon hydroxylase activities by monoclonal antibodies. *Dev Pharmacol Ther* **10**: 125–132, 1987.
34. Pacifici GM, Park SS, Gelboin HV and Rane A, 7-Ethoxycoumarin and 7-ethoxyresorufin *O*-deethylase in human foetal and adult liver: studies with monoclonal antibodies. *Pharmacol Toxicol* **63**: 26–29, 1988.
35. Pelkonen O, Sotaniemi EA and Ahokas JT, Coumarin 7-hydroxylase activity in human liver microsomes. Properties of the enzyme and interspecies comparisons. *Br J Clin Pharmacol* **19**: 59–66, 1985.
36. Mäenpää J, Pelkonen O, Cresteil T and Rane A, The role of cytochrome P450 3A (CYP3A) isoform(s) in oxidative metabolism of testosterone and benzphetamine in human adult and fetal liver. *J Steroid Biochem Mol Biol*, in press.
37. Crespi CL, Penman PW, Leakey JAE, Arlotto MP, Stark A, Parkinson A, Turner T, Steimel DT, Rudo K, Davies RL and Langenbach R, Human cytochrome P450IIA3: cDNA sequence, role of the enzyme in the metabolic activation of promutagens, comparison to nitrosamine activation by human cytochrome P450IIE1. *Carcinogenesis* **11**: 1293–1300, 1990.
38. Maurice M, Emiliani S, Dalet-Beluche I, Derancourt J and Lange R, Isolation and characterization of a cytochrome P450 of the IIA subfamily from human liver microsomes. *Eur J Biochem* **200**: 511–517, 1991.
39. Miles JS, Spurr NK, Gough NC, Jowett T, McLaren AW, Brook JD and Wolf CR, A novel human cytochrome P450 gene (P450IIB): chromosomal localization and evidence for alternative splicing. *Nucleic Acids Res* **16**: 5783–5795, 1988.
40. Yamano S, Nhamburo PT, Aoyama T, Meyer UA, Inaba T, Kalow W, Gelboin HV, McBride OW and Gonzalez FJ, cDNA cloning and sequence and cDNA-directed expression of human P450IIB1: identification of a normal and two variant cDNAs derived from the CYP2B locus on chromosome 19 and differential expression of the IIB mRNAs in human liver. *Biochemistry* **28**: 7340–7348, 1989.
41. Wrighton SA, Vandenbranden M, Becker GW, Black SD and Thomas PE, Two monoclonal antibodies recognizing different epitopes on rat cytochrome IIB1 react with human IIE1. *Mol Pharmacol* **41**: 76–82, 1992.
42. Sesardic D, Pasanen M, Pelkonen O and Boobis AR, Differential expression and regulation of members of the cytochrome P450IA gene subfamily in human tissues. *Carcinogenesis* **11**: 1183–1188, 1990.
43. Murray GI, Foster CO, Barnes TS, Weaver RJ, Snyder CP, Ewen SWB, Melvin WT and Burke MD,

Cytochrome P450IA expression in adult and fetal human liver. *Carcinogenesis* **13**: 165–169, 1992.
44. Gelboin HV and Friedman FK, Monoclonal antibodies for studies on xenobiotic and endobiotic metabolism. Cytochromes P-450 as paradigm. *Biochem Pharmacol* **34**: 2225–2234, 1985.
45. Romkes M, Faletto MB, Blaisdell JA, Raucy JL and Goldstein JA, Cloning and expression of complementary DNAs for multiple members of the human cytochrome P450IIC subfamily. *Biochemistry* **30**: 3247–3255, 1991.
46. Pons C, Dansette PM, Amar C, Jaouen M, Wolf CR, Gregeois J, Homberg JC and Mansuy D, Detection of human hepatitis anti-liver kidney microsomes (LKM2) autoantibodies on rat liver sections is predominantly due to reactivity with rat liver P-450 IIC11. *J Pharmacol Exp Ther* **259**: 1328–1334, 1991.
47. Doecke CJ, Sansom LN and McManus ME, Phenytoin 4-hydroxylation by rabbit liver P450IIC3 and identification of orthologs in human liver microsomes. *Biochem Biophys Res Commun* **166**: 860–866, 1990.
48. Gut J, Meier UT, Catin T and Meyer UA, Mephenytoin-type polymorphism of drug oxidation: purification and characterization of a human liver cytochrome P-450 isozyme catalyzing microsomal mephenytoin hydroxylation. *Biochim Biophys Acta* **884**: 435–447, 1986.
49. Shimada T, Misono KS and Guengerich FP, Human liver microsomal cytochrome P-450 mephenytoin 4-hydroxylase, a prototype of genetic polymorphism in oxidative drug metabolism: purification and characterization of two similar forms involved in the reaction. *J Biol Chem* **261**: 909–921, 1986.
50. Leo MA, Lasker JM, Raucy JL, Kim C-I, Black M and Lieber CS, Metabolism of retinol and retinoic acid by human liver cytochrome P450IIC8. *Arch Biochem Biophys* **269**: 305–312, 1989.
51. Knodell RG, Dubey RK, Wilkinson GR and Guengerich FP, Oxidative metabolism of hexobarbital in human liver: relationship to polymorphic *S*-mephenytoin 4-hydroxylation. *J Pharmacol Exp Ther* **245**: 845–849, 1988.
52. Umbenhauer DR, Martin MV, Lloyd RS and Guengerich FP, Cloning and sequence determination of a complementary DNA related to human liver microsomal cytochrome P-450 *S*-mephenytoin 4-hydroxylase. *Biochemistry* **26**: 1094–1099, 1987.
53. Bhamre S, Anandatheerthavarada HK, Shankar SK and Ravindranath V, Microsomal cytochrome P450 in human brain regions. *Biochem Pharmacol* **44**: 1223–1225, 1992.
54. Bornheim LM and Correia MA, Purification and characterization of the major hepatic cannabinoid hydroxylase in the mouse: a possible member of the cytochrome P-450IIC subfamily. *Mol Pharmacol* **40**: 228–234, 1991.
55. Wrighton SA, Thomas PE, Willis P, Maines SL, Watkins PB, Levin W and Guzelian PS, Purification of a human liver cytochrome P-450 immunochemically related to several cytochromes P-450 purified from untreated rats. *J Clin Invest* **80**: 1017–1022, 1987.