Temporal expression profiles of organic anion transport proteins in placenta and fetal liver of the rat


1 Division of Clinical Pharmacology and Toxicology, Department of Medicine, and 2 Department of Pathology, University Hospital, Zürich 8091, Switzerland; 3 Institute of Clinical Pharmacology, Inselspital, University of Berne, 3010 Berne, Switzerland; 4 Department of Biochemistry and Molecular Biology and 5 Department of Physiology and Pharmacology, University of Salamanca, Salamanca, Spain; and 6 Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan

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The liver is equipped with a host of transport systems that mediate the uptake and secretion of endogenous and exogenous organic anions. In the rat, the Na+/taurocholate cotransporting polypeptide (NTCP) (Slc10a1) at the basolateral surface of hepatocytes (17) and the bile salt export pump (Bsep) (Abcb11) at the canalicular membrane (10) govern the hepatocellular uptake of anionic endogenous solutes and xenobiotics (50). The multidrug resistance protein (MRP) family, Oat2 (Slc28a2), is also abundantly expressed basolaterally (50). The multidrug resistance protein (MRP) family of pumps, which are classified within the ATP binding cassette (ABC) gene superfamily of transport proteins (20, 26), export anionic endogenous solutes and xenobiotics from the hepatocyte. MRP2 (Abcc2) resides at the canalicular membrane and secretes its substrates into bile (43). MRP3 (Abcc3) is present at low levels at the basolateral membrane for export of substrates into sinusoidal blood and is upregulated, along with MRP1 (Abcc1), when MRP2 is downregulated (56) and in some cases of cholestasis (42, 51). MRP6 (Abcc6), for which few substrates have been identified, is confined to the lateral surface of rat hepatocytes (34).

The ontogenetic patterns of many transporters have been studied to elucidate the molecular basis of neonatal cholestasis, a well-known phenomenon in both rat and human neonates, especially in preterm births (27, 55). In the rat model, the finding that RNA and protein levels of Ntcp, Oatp1a1, Oatp1a4, MRP2, and Bsep can take up to several weeks to reach full adult expression (2, 9, 14, 18, 62) has linked the delayed development of hepatobiliary organic anion transport systems to the physiological cholestasis that occurs at birth. Yet the biosynthesis of organic anions, such as bilirubin, bile salts, and steroid sulfates, normally cleared by the liver, begins in utero. At this time, the fetus must rely, in part, on the placenta for the elimination of fetal-derived solutes. Indeed, experimental evidence shows that the placenta directs the vectorial transfer of organic anionic compounds toward the maternal circulation for

Address for reprint requests and other correspondence: Marie V. St-Pierre, Div. of Clinical Pharmacology and Toxicology, Univ. Hospital Zürich, 100 Rämistrasse, Zürich 8091, Switzerland (E-mail: stpierre@kpt.unizh.ch).

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excretion (4, 36, 48) and the placenta does express some of the organic anion transporters in common with the liver (29, 49, 53, 54). However, the expression levels in the placenta have not been evaluated in relation to time of gestation or to the corresponding levels in the fetal liver, and hence the relative importance of the placenta as an excretory organ for the developing fetus has not been established. In addition, it is not known whether transporters in the placenta are subject to the same nuclear receptor-mediated regulatory mechanisms that operate in the liver to modulate expression levels. Several endogenous as well as exogenous substances serve as ligands for the nuclear receptors, pregnane X receptor (PXR) and constitutive androstane receptor (CAR) (12, 28, 30). Both Oatp1a4 and Mrp2 are target genes for PXR in vitro (15, 24), whereas Mrp2 and Mrp3 may be regulated by a CAR-based mechanism in rat liver (24, 58).

We hypothesized that organic anion transporters are expressed in the rat placenta and fetal liver in a coordinated manner that allows the placenta to compensate for the late maturation of transporters displayed in the fetal liver. Furthermore, we hypothesized that the placenta is equipped with PXR- and CAR-based regulatory mechanisms for transporter expression. To test these two hypotheses, we compared the temporal gene expression profiles of organic anion transport proteins in the placenta and fetal liver of the rat, as well as the relative expression levels of PXR and CAR.

**MATERIALS AND METHODS**

**RNA Isolation and cDNA Synthesis**

Female Wistar rats (170–200 g) were obtained from the Institut für Labor-Tierkunde, University of Zürich, and cared for in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). Estimates of the age of embryos were based on timed matings. Immediately after killing the animal, total RNA was extracted by the Trizol method (Life Technologies, Rockville, MD) from fetel livers, beginning at day 15.5, a point at which the organ is easily dissectable and at which intrapheatic bile ducts have begun to differentiate and at days 17, 19, 20, and 21.5. Fetuses from each litter were pooled with no further differentiation between male and female offspring. Isolation of total RNA from placentas began earlier at days 13, 15.5, 17, 19, 20, and 21.5. Random hexamer primed first-strand cDNA synthesis was performed with 2 μg RNA in the presence and absence of reverse transcriptase 125 U (MultiScribe, Applied Biosystems, Foster City, CA).

**Real-Time RT-PCR: Relative Quantification**

A real-time quantitative RT-PCR assay was chosen because it offered increased sensitivity and a broader linear dynamic range than conventional Northern analysis. The RNA expression of the following transporters was characterized in fetal rat liver and placenta: Oatp1a1, Oatp1a4, Oatp1a5, Oatp2b1, Oatp4a1, Oat2, Mrp1, Mrp2, Mrp3, Mrp6, Ntcp, Bsep. Gene-specific primers and 5′-(FAM)-(TAMRA)-3′ labeled probes were designed with Primer Express (Applied Biosystems, Foster City, CA) software for use in the real-time RT-PCR assay (Taqman). The primer and probe set for each carrier is listed in Table 1. The primer probe set for PXR was forward 5′-taggacagctgtcacagcaaa, reverse 5′-gcagactttgcccaccatagc, probe 5′-attctcgactgcgttggctga. The primer probe set designed for CAR was forward 5′-catggtgaccaggcttgctg, reverse 5′-agacggagagagagag, probe 5′-ctaatgctctctgcttcgat.

The known intron-exon boundary information for each target gene was taken into account to avoid detection of contaminating genomic DNA. The probes for the cDNAs of Oatp1a1, Oatp1a5, Oatp2b1, Oatp4a1, Oat2, Mrp1, Mrp2, Mrp3, Mrp6, PXR, and CAR spanned an intron-exon junction (Table 1). The reverse primer was positioned over the intron-exon boundary.

Table 1. Primers and 5′-(FAM)-labeled probes designed for quantitative real-time PCR measurement of carrier expression

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<th>Carrier (Gene)*</th>
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<th>Probe</th>
<th>Reverse Primer</th>
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Ntcp, Na+/taurocholate cotransporting polypeptide; Oatp, organic anion transporting polypeptide; Bsep, bile salt export pump; Mrp, multidrug resistance protein. * Indicates the position of the predicted intron-exon boundary; † The gene symbols in parentheses are consistent with the Human Gene Nomenclature Committee Database.
for Oatp1b2 and Ntcp, whereas the probe and reverse primer were on different exons for Oatp1a4 and Bsep. The specificity of each primer pair was verified by sequencing the amplified PCR product. In addition, for the closely related Oat gene family, all primer pair-probe combinations were tested for amplification with Oatp1a1, Oatp1a4, Oatp1a5, Oatp1b2, and Oatp1b6 cDNAs. Reactions (20–40 ng transcribed RNA) were performed using Taqman universal PCR master mix with 300 nmol/l forward and reverse oligonucleotide primers and 200 nmol/l FAM fluorescent dye-labeled probe on an ABI Perkin Elmer Prism 7700 Sequence Detection System (Applied Biosystems). For the purpose of relative quantification, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control reference. Each reaction was measured in triplicate in the placentas and the pooled fetal livers of three rats at each gestational time point. Data are expressed as means ± SD. Analysis of the fluorescent data yielded Ct values, defined as the number of PCR cycles required for the signal to exceed the detection threshold value using the Sequence Detection System software (Applied Biosystems). The linear range of the fluorescence signal on a 1,000-fold amplification range in target transcripts (log_{10} ng transcribed RNA vs. CT) was assessed. The relative efficiency of the PCR amplifications for endogenous reference and target genes was also validated by comparing the efficiency of the PCR amplifications for endogenous reference, a 315-bp fragment was amplified from rat placenta using the 18S PCR primer pair supplied with the QuantumRNA 18S Internal Standards kit (Ambion) and cloned in the pCR4 vector.

cRNA synthesis. Individual cRNAs were synthesized with the MEGAscript T7 or T3 kit (Ambion) using the corresponding full-length cDNAs as template, except for the 18S ribosomal RNA where the 315-bp PCR transcript served as the template. The cRNA transcripts were purified with the MEGAclear kit (Ambion), loaded onto a denaturing formaldehyde agarose gel to verify the quality and size of the products and then quantitated by measuring absorbance at 260 nm.

Standard curves. The mass (in μg) of cRNA standards was converted to copy number as follows:

\[ \text{number of copies} = \frac{6.02 \times 10^{23} \times \text{μg cRNA}}{340.5 \times \text{number of bases}} \]

where 340.5 is the molecular weight of 1 ribo base.

Serial dilutions of each cRNA were made over eight orders of magnitude to give standard solutions containing 1.02 × 10^{-6} ng to 0.40 ng per 100 μl. Serial dilutions of the 18S ribosomal cRNA standard solution ranged from 100 ng to 6.4 × 10^{-3} ng per 100 μl. Reverse transcription reactions were performed in duplicate with 2.5 μM random hexamers and Multiscribe reverse transcriptase enzyme. To approximate the heterogeneous composition of the experimental material where the bulk of the total RNA consists of rRNA, 1 μg of the 18S ribosomal cRNA fragment was added to all the cRNA standard solutions before reverse transcriptase and amplification. Aliquots of 5 μl were taken for amplification, in triplicate, by real-time PCR using the primers and probes listed (Table 1). The FAM-TAMRA-labeled probe used in the quantification of 18S ribosomal RNA was 5'-TGGCTGAACGCCACTTTGCTCCCCCTTAA-3', which hybridized to the 1449–1474 bp position of the rat gene. To compensate for the abundance of the rRNA in the experimental samples compared with the target mRNA of the transporters, it was necessary to reduce the PCR amplification efficiency of the 18S cDNA template and bring the Ct signal of the 18S cDNA endogenous reference into the same linear range as that of the target transporters. To accomplish this, the 18S rRNA primer/competimer combination supplied with QuantumRNA 18S Internal Standards kit (Ambion) was added to the Taqman PCR mixture in a ratio of 3:7, in lieu of the primers alone.

Standard curves were generated by plotting the Ct values given by the real-time PCR assay against the logarithm of the initial copy number (S). The target copy numbers of each individual transporter per microgram total RNA was calculated from the linear regression of the corresponding standard curve and then normalized to the calculated copy number of 18S ribosomal RNA in the same sample.

Immunodetection of Mrp1 and Mrp3 in the Placenta

Placentas were dissected free of fetal membranes and decidua and immediately frozen in isopentane precooled in liquid nitrogen and stored at −80°C before cutting sections (10 μm) on a cryomicrotome at −20°C. To detect Mrp1, sections were fixed in acetone for 8 min,
rehydrated in PBS, blocked with 3% normal goat serum (Dako) and Triton 0.05% diluted in Tris-buffered saline (TBS), and incubated for 2 h with a polyclonal antibody Mrp1 (A23) (Alexis Biochemicals) diluted to 1:40 in blocking solution. Sections were then washed and incubated for 30 min with Cy2-conjugated F(ab’2) fragment goat anti-rabbit IgG (Jackson Immuno-Research, West Grove, PA). For double-labeled immunofluorescence, the incubation mixture also included monoclonal mouse antibodies against vimentin (clone V9) (Neomarkers, Fremont CA), CD31 (Pharmingen, Germany), or P-glycoprotein (C219) (Alexis Biochemicals), which were detected using a Cy5-conjugated goat anti-mouse IgG as a secondary antibody. Nuclei were counterstained for 2 min in 1 mmol/l 4’-6-diamidino-2-phenylindole (DAPI). To detect Mrp3, tissue sections were fixed in 3% paraformaldehyde and processed as described by Soroka et al. (51) and then incubated with the anti-Mrp3 polyclonal antibody (R1508 ORGANIC ANION TRANSPORTERS IN RAT PLACENTA). The normalized levels of Oatp1a5 mRNA in the placenta exceeded those in the fetal liver until day 15 (Fig. 1E). In contrast to Oatp1a1, Oatp1a4 mRNA was not consistently detected in all placenta samples and first appeared in the fetal liver 1 day before birth (Fig. 1A). The normalized levels of Oatp1a5 mRNA in the placenta approached 100%, indicating equal expression of this carrier in the placenta and adult female liver (Fig. 1B), but levels were nevertheless very low in both organs. Oatp1a5 transcripts were undetectable in the fetal liver. The expression of Oatp1b2 mRNA in placenta was lower than in the fetal liver and decreased toward the end of gestation (Fig. 1B, Table 2), from $3 \times 10^{-7}$ copies per copy of 18S rRNA at day 13 to $6 \times 10^{-8}$ at day 19 and beyond. In contrast, the placent al expression of Oatp2b1 was higher and increased by one order of magnitude, from $5 \times 10^{-7}$ copies per copy of 18S rRNA at day 15 to $4 \times 10^{-6}$ at day 20 of gestation. The levels in placenta exceeded those in the fetal liver until day 20 (Fig. 1C; Table 2). Similarly, the placent al levels of Oatp4a1 mRNA increased throughout gestation (Fig. 1D) and were the most abundant Oatp measured in the placenta (Table 2). Conversely, Oatp4a1 was the least abundant transporter detected in the liver. The mRNA levels of Oat2 in the placenta reached 0.01% or less of those in the maternal liver and were always significantly

**RESULTS**

To assess whether the placent al expression of organic anion carriers changed during gestation and to gauge the abundance of each carrier in the placenta relative to the fetal liver, the real-time PCR data from both the placenta and fetal livers were normalized to the mean levels measured in female adult livers. Data were plotted on a logarithmic scale to visualize the breadth of the concentration ranges measured over time in the two organs (Figs. 1–3). For a more critical assessment of the abundance of individual transporters in the placenta, the absolute quantification of the mRNA copy number was performed for each transporter whose levels reached at least 0.1% of those measured in the adult female liver. The mRNA copy numbers were normalized to the endogenous reference, 18S rRNA (Table 2).

Oatp1a1 mRNA was detectable in the placenta at low levels throughout gestation and before its appearance in the fetal liver at gestational day 19 (Fig. 1A). In the placenta, the absolute copy number fluctuated between $1.7 \times 10^{-9}$ and $5.9 \times 10^{-8}$ copies per copy of 18S rRNA (Table 2). In contrast to Oatp1a1, Oatp1a4 mRNA was not consistently detected in all placenta samples and first appeared in the fetal liver 1 day before birth (Fig. 1A). The normalized levels of Oatp1a5 mRNA in the placenta approached 100%, indicating equal expression of this carrier in the placenta and adult female liver (Fig. 1B), but levels were nevertheless very low in both organs. Oatp1a5 transcripts were undetectable in the fetal liver. The expression of Oatp1b2 mRNA in placenta was lower than in the fetal liver and decreased toward the end of gestation (Fig. 1B, Table 2), from $3 \times 10^{-7}$ copies per copy of 18S rRNA at day 13 to $6 \times 10^{-8}$ at day 19 and beyond. In contrast, the placent al expression of Oatp2b1 was higher and increased by one order of magnitude, from $5 \times 10^{-7}$ copies per copy of 18S rRNA at day 15 to $4 \times 10^{-6}$ at day 20 of gestation. The levels in placenta exceeded those in the fetal liver until day 20 (Fig. 1C; Table 2). Similarly, the placent al levels of Oatp4a1 mRNA increased throughout gestation (Fig. 1D) and were the most abundant Oatp measured in the placenta (Table 2). Conversely, Oatp4a1 was the least abundant transporter detected in the liver. The mRNA levels of Oat2 in the placenta reached 0.01% or less of those in the maternal liver and were always significantly

**A** Oatp1a1 and Oatp1a4 mRNA (% of Adult Liver) (A)

**B** Oatp1a5 and Oatp1b2 mRNA (% of Adult Liver) (B)

**C** Oatp2b1 mRNA (% of Adult Liver) (C)

**D** Oatp4a1 mRNA (% of Adult Liver) (D)

Fig. 1. Normalized expression of organic anion transporting polypeptide (Oatp) mRNA in the fetal liver and placenta of rats as a function of gestational age measured by quantitative real-time PCR. Solid symbols, placenta; open symbols, fetal liver. A: Oatp1a1 in the fetal liver (○) and Oatp1a4 in the fetal liver (○) and placenta (△) quantified using GAPDH as the endogenous reference. B: Oatp1a5 in the placenta (△) and Oatp1b2 in the fetal liver (○) and placenta (△). C: Oatp2b1 in the fetal liver (○) and the placenta (○). D: Oatp4a1 in the fetal liver (○) and the placenta (○). Data are expressed as a percentage of the mRNA levels measured in the adult female liver. Measurements were performed in triplicate in 3 rats at each gestational time point and are presented as means ± SD.
Table 2. Absolute quantification of transporter gene expression in the rat placenta throughout gestation
No. of copies of mRNA transcripts per copy number of 18S ribosomal RNA (SD)

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<th>Day 20</th>
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<td>4.9×10^{-7}</td>
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<td>(2.9×10^{-8})</td>
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<td>(3.8×10^{-8})</td>
<td>(1.1×10^{-6})</td>
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The copy numbers of individual mRNA transcripts were determined in triplicate in the placentae of 3 rats at the indicated times of gestation and in the adult female liver. Copy numbers of each transporter were normalized for the copy number of 18S ribosomal RNA. External standard curves were generated using serial dilutions of each cRNA (0.40 ng down to 1.02×10^{-6} ng/100 μl) as template for reverse transcription and real-time PCR. The Ct values obtained were plotted against the logarithm of the initial copy number of the cRNA standard. ND = not detected.

less than in the fetal liver (Fig. 2A). Similarly, Ntcp was detectable in all placenta samples at 0.01% or less than in the adult female liver and always less than in the fetal liver (Fig. 2B). The placental profile of Bsep mRNA was constant at 1% of the adult liver and surpassed the fetal liver levels until the 19th day of gestation (Fig. 2C). The absolute copy number of Bsep showed it to be of low abundance (Table 2).

The MRP efflux pumps showed different expression trends than the uptake carriers. The placental expression of Mrp1 was 10–20 times higher than in the developing liver, a consistent

Fig. 2. Normalized expression of the mRNA of Oat2 and the bile salt transporters Na^-taurocholate cotransporting polypeptide (Ntcp) and bile salt export pump (Bsep), in the fetal liver and placenta of rats as a function of gestational age, measured by quantitative real-time PCR. A: Oat2 in the fetal liver (○) and the placenta (●). B: Ntcp in the fetal liver (○) and the placenta (●). C: Bsep in the fetal liver (○) and the placenta (●). Data are expressed as a percentage of the mRNA levels measured in the adult female liver. Measurements were performed in triplicate in 3 rats at each gestational time point and are presented as means ± SD.
finding throughout gestation. Mrp1 mRNA levels in the fetal liver were low but two- to threefold higher than in the adult female liver (Fig. 3A). Transcripts of Mrp1 were the most abundant of all Mrp members tested and were approximately equal to those of Oatp4a1 (Table 2). Mrp2 RNA was always detectable in the placenta, but its level varied from 0.2 to 1% of the adult female liver levels and was less than in the corresponding fetal liver (Fig. 3B). The absolute copy numbers ranged from one to two orders of magnitude lower than those of Mrp1. The placental levels of Mrp3 were approximately equal to adult liver levels throughout gestation. The absolute copy numbers were intermediate between those of Mrp1 and Mrp2 (Table 2). The fetal liver expression of Mrp3 was ~10% of the adult levels until a surge at day 20, when it increased to adult levels (Fig. 3C). Mrp6 was barely detectable in the placenta, with levels not reaching 0.1% of the adult levels in the liver (Fig. 3D). However, as with all other members of the Mrp family, it was expressed early in the fetal liver and reached 10% of adult levels when first measured at day 15.

Immunolocalization studies of Mrp1 in the placenta were performed as a means of understanding its role in the efflux of natural ligands and xenobiotics in gestation. A schematic representation of the rat placenta outlines the major structures (Fig. 4A). In near-term placentas (day 20), Mrp1 was abundantly expressed in the layers of cells surrounding large fetal vessels located within placental labyrinth (Fig. 4B). These are choioallantoic vessels that arise from the extraembryonic allantois and advance into the placental labyrinth (Fig. 4A) (11). The Mrp1-expressing cells are endodermal epithelial cells; they line the yolk sac diverticula that accompany the choioallantoic vessels. The lumen of yolk sac diverticula is continuous with the larger yolk sac cavity (Fig. 4A). Concomitant immunostaining for P-glycoprotein served to mark the labyrinth zone, specifically the apical side of one or both layers of syncytiotrophoblast, facing the maternal blood spaces (Fig. 4B). There was occasional faint staining for Mrp1 in the syncytiotrophoblast layer, but this did not coincide with P-glycoprotein (Fig. 4B). To confirm that the Mrp1 protein was expressed at comparable levels at earlier gestational times as indicated by the mRNA measurements, placentas from day 15.5 were examined (Fig. 4, C and D). At this time, the collections of choioallantoic vessels were embedded within a larger mesodermal mass that entered the labyrinth and stained positively for vimentin, a marker for tissues of mesenchymal origin (Fig. 4C). The Mrp1-expressing cells were negative for vimentin. To differentiate blood vessels from the surrounding mesenchymal structures, double-labeling immunofluorescence detected the cell adhesion molecule, CD31 (Fig. 4D). Only the choioallantoic vessels and the fetal capillaries of the labyrinth zone gave positive signals for CD31. Mrp1 was not expressed in endothelial cells. No Mrp1 was detected in the syncytiotrophoblast layers of placentas before day 20. Mrp3 was also immunolocalized. The faint signals for Mrp3 compared with Mrp1 were consistent with the lower expression of Mrp3 transcripts (Table 2). The most prominent immunostaining appeared along the apical membranes of the endodermal epithelial cells that line the yolk sac diverticula that were previously identified as Mrp1-expressing cells (Fig. 5A). A very
faint signal was also detected in the syncytiotrophoblast layers of the labyrinth zone (Fig. 5B). When the antibody staining was visualized under light microscopy using a chromogen, the syncytiotrophoblast staining was clearer (Fig. 5D). No signals coincided with endothelial cells lining the fetal capillaries, but weak staining was detected in the endothelium of the maternal feeder arteries (Fig. 5E).

The placenta consistently expressed very low levels of PXR, where normalized values were 0.01% to 0.1% of the adult female liver (Fig. 6A). In the fetal rat liver the mRNA levels rose swiftly, from 3.6 ± 0.4% of adult female liver at day 15.5 to 64 ± 9% at birth. Similarly, the placental mRNA expression of CAR was low, ranging from 0.5% to 0.9% of the adult female liver levels (Fig. 6B). In the fetal liver, CAR was 10% of the adult female liver at day 15.5 and rose sharply at day 20 to equal the level in the adult female liver.

**DISCUSSION**

The mRNA levels of organic anion carriers in the placenta and fetal liver of the rat were compared as a function of gestational age to gauge the relative abundance of transporter expression in the placenta and to determine whether the placenta displays a compensatory mechanism for the delayed maturation that affects several transporters in the developing liver. Our findings show that the placenta is equipped with a unique set of organic anion transporters, and we identify those most abundant throughout gestation. Moreover, the temporal
mRNA profiles of one member of the Oatp superfamily, one member of the Mrp family, and Bsep implicate these transporters in placental excretion early in gestation when their expression in the developing liver remains low.

Oatp2b1 emerges as a major uptake carrier in rat placenta based on its mRNA expression that increased during gestation and exceeded that of the fetal liver up until day 20 and based on the abundance of its copy number (Table 2). Although Oatp2b1 has not yet been immunolocalized in the rat placenta, its human ortholog, OATP2B1 (77% identity), is expressed at the basal (fetal facing) surface of the syncytiotrophoblast where it is positioned to transfer fetal-derived anionic solutes, such as estrone-3-sulfate and dehydroepiandrosterone sulfate, across the placenta in the fetal-maternal direction (53). The list of natural substrates identified for the rat Oatp2b1 differs from the human carrier: leukotriene C₄ exhibited the highest signal in uptake assays and taurocholate and selected prostaglandins gave smaller signals (40). Because the Oatp2b1 mRNA in the fetal liver had already reached 1% of adult levels at day 15.5 and increased to 11% at day 20 of gestation (Fig. 1C), Oatp2b1 may also be operative in utero, unlike the Oatp1a1, Oatp1a4, and Oatp1b2 proteins, which cannot be immunodetected (9). It is reasonable then to postulate that Oatp2b1 clears endogenous anions from the fetal circulation by uptake into the fetal liver as well as transfer across the placenta in the fetal-maternal direction and that the placental transfer predominates in early gestation. Oatp4a1 transcripts were the most abundant of all uptake carriers in rat placenta. This expression was unique to the placenta because the fetal and adult liver levels, although detectable, were very low (Table 2). Despite this abundance, the role of the Oatp4a1 protein in placental physiology remains unclear. Substrates identified for both Oatp4a1 and its human ortholog, OATP4A1 (76% identity), include taurocholate, 3′-5′-3′-l-triiodothyronine, and prostaglandin E₂ (8).

Among the members of the Mrp family, Mrp1 emerges as a major export pump in the placenta that may act in concert with the fetal liver to dispose of fetal-derived solutes in utero. The Mrp1 protein is normally undetectable in adult rat liver but in the fetal liver, mRNA levels were two to three times higher than those measured in the adult liver (Fig. 3A). In the placenta, Mrp1 transcripts were the most abundant of all export pumps investigated (Table 2). Mrp1 serves a long list of natural
ligands, including sulfated steroids, as well as the glutathione S-conjugate of 4-hydroxynonenal, leukotriene C4 and glutathione disulfide, products formed during oxidative stress (46). There is evidence linking fetal hepatocytes to a predisposition to oxidative damage, partly due to low antioxidant enzymes (19), and Mrp1 itself is induced under conditions of oxidative stress (59). The fetal liver may rely on Mrp1 for extrusion of these products from hepatocytes into the circulation. In the rat placenta, the Mrp1-expressing epithelial cells that are in close proximity to the fetal chorioallantoic vessels are then in a position to export fetal-derived solutes into the lumen of the yolk sac diverticula, which is continuous with the yolk sac cavity (Fig. 4). This could provide a mechanism whereby metabolic end products or other Mrp1 substrates are sequestered from both the maternal and fetal circulations throughout gestation.

The physiological significance of Mrp2 in the rat placenta is less apparent. The mRNA levels were low relative to Mrp1 and Mrp3 (Table 2) and far below the levels measured in the fetal liver (Fig. 3B), and the Mrp2 protein was below the limits of immunodetection (data not shown). Conversely, the fetal rat liver showed early expression of Mrp2 mRNA (Fig. 3B) and protein at the canalicular domain of hepatocytes (9), suggesting that the fetus does not rely on placental excretion of Mrp2 substrates in utero. Nevertheless, the Mrp2 protein has been detected in rat placenta by Western blotting after treatment with ursodeoxycholic acid for obstructive cholestasis (49). This raises the possibility that placental Mrp2 is upregulated in cases of maternal hepatic disease associated with the systemic accumulation of solutes toxic to the fetus. The human MRP2 has previously been localized to the apical surface of the human syncytiotrophoblast (54), which is consistent with a role in protecting the fetal compartment against entry of numerous MRP2 substrates, including leukotrienes, the sulfate and glucuronide conjugates of steroids, sulfated bile salts, and products of oxidative stress (3, 25, 45).

The profile of Mrp3 in the placenta suggests a role in gestation (Figs. 3C and 5). Mrp3 exports a range of sulfated and glucuronidated conjugates and is a low-affinity transporter of bile salts, including dianionic sulfated bile salts (21). The existence of an ATP-dependent transport system in the apical (maternal facing) syncytiotrophoblast membranes that mediate the vectorial, fetal-to-maternal export of bile salts, has been shown unequivocally (4, 32). The faint staining of Mrp3 in the syncytiotrophoblast layers of the labyrinth makes it a candidate protein for this function, although an apical sublocalization could not be confirmed in our studies. A stronger Mrp3 expression was detected in the epithelial cells surrounding the yolk sac diverticula, as was the case with Mrp1, which again suggests that export into the yolk sac diverticula is part of the excretory pathway in the rat placenta. The occasional staining of Mrp3 associated with the endothelium of the large maternal feeder arteries reflects an even broader role in gestational tissue, by restricting access of solutes to the maternal blood compartment if Mrp3 resides abuminally or excreting solutes into the maternal blood if Mrp3 is expressed on the luminal side.

The mRNA expression of Bsep in placenta was 10-fold lower than for Mrp3, which implies that its participation in the clearance of fetal bile salts across the placenta is less important (Table 2). Nevertheless, placental mRNA greatly exceeded expression in the fetal liver until gestational day 20. During this time in development there is minimal expression of cholesterol 7α-hydroxylase (37), resulting in very low levels of fetal bile acids whose elimination may be adequately handled by Bsep-mediated placental transport. After day 20, mRNA expression of Bsep in the fetal liver quickly outpaced that in the placenta (Fig. 2C), at which point the Bsep protein is known to be detectable at the canalicular membranes of the fetal liver (9). This onset of canalicular bile acid secretion coincides with the increase in bile acid pool size (31), which in turn suggests that the ontogenesis of Bsep in the fetal liver keeps pace with the gestational needs of the fetus. The unlikely role for Bsep as the main transporter of fetal bile acids late in gestation is also supported by the high $K_m$ value (120 μmol/l) reported for ATP-dependent transport in isolated placental membrane vesicles of term rat placentas (49), which greatly exceeds the expected value for the Bsep protein (10, 41).

Ntcp, oat, and the oatp members, oatp1a1, oatp1a4, oatp1a5, and oatp1b2, were judged to make a minor contribution to the overall transfer of organic anions across the rat placenta. Ntcp expression in the placenta was very low (Table 2), whereas the exponential rise in mRNA expression in the fetal liver from day 15.5 onward (Fig. 2B) can explain its early appearance at the basolateral membrane at day 20 (9) and confirms that the ontogenesis of hepatocytic uptake and cana-
licular secretion of bile salts are coordinated. In the case of Oat2, not only the fetal liver (Fig. 2A) but also the fetal kidneys and lung express this carrier in utero (44), whereas it is barely detectable in the placenta. For oatp1b2, the declining mRNA expression in the placenta relative to the fetal liver argues against oatp1b2-mediated clearance of substrates by the placenta in utero. Likewise, placental expression does not compensate for the protracted ontogenetic liver profiles of oatp1a1 and oatp1a4 (Fig. 1) (2, 9), although these transporters could become relevant when upregulated in vivo (49). The placental mRNA expression of oatp1a5 equals that of the adult female liver, but there it is poorly expressed and not immunodetectable (57). Oatp1a1, oatp1a4, and oatp1a5 mediate the uptake of a broad range of common substrates (16), and despite their minimal expression in fetal liver or placenta, other tissues such as the kidney for oatp1a1 (13) and the intestine for oatp1a5 (57) may express these carriers prenatally.

Multispecific organic anion transporters expressed in the placenta will partly determine the extent of fetal exposure to anionic drugs and toxins ingested by the mother, a process that may be more critical to predict early in gestation. The rodent Mrp2 protein, the oatp1a4 mRNA and protein, and the mdr genes that code for P-glycoprotein can be upregulated by mechanisms involving the xenobiotic sensing transcription factor, PXR (15, 23, 35, 52). Mrp2 and Mrp3 are inducible by xenobiotics in part by a CAR-based mechanism (7, 24, 58). RT-PCR studies reporting that PXR was expressed in mouse placenta (38) prompted us to include PXR and CAR in our investigations. PXR and CAR were minimally expressed in rat placenta relative to the fetal liver (Fig. 6), implying that the placenta is ill-equipped to respond to certain drugs/toxins in the maternal circulation by upregulating target genes by these means. However, the fact that specific transporters can be induced experimentally (49) indicates that alternative mechanisms are in place to allow the placenta to enhance its clearance role on demand. The profile of CAR in the fetal rat liver, normalized to the levels measured in the female adult liver (Fig. 6), does not agree with the ontogenesis reported in mice or with the decreased mRNA and protein expression of CAR documented in human neonatal livers (22). The recognition that in rat liver, CAR expression is strain and gender specific can help explain this discrepancy (61). A previous study showed that the CAR protein was undetectable in total fetal liver extracts of female Wistar rats (61), and we would argue that normalization of fetal liver RNA transcripts to the low levels of CAR expressed in female Wistar rat livers resulted in high apparent yields that predicted an abrupt increase from 10% prenatally to 100% of adult liver levels at birth (Fig. 6). A different ontogenetic profile would most likely emerge in another strain or if male rat livers were used for normalization. The fact that in the previous study, data generated by RT-PCR failed to show these major differences in CAR mRNA when strains and genders of rats were compared may simply reflect the nonquantitative limitation of conventional RT-PCR (61).

Our experiments permit a critical interpretation of the mRNA levels of transporters in rat placenta and complement recent studies (29, 49). The relative quantification comparing the fetal liver and placenta used gapdh as an endogenous reference. The absolute quantification that gave the precise copy number of individual mRNA transcripts per microgram of RNA relied on 18s rRNA. There are reports critical of the use of gapdh, because of variable expression during development (5). In our hands, gapdh proved to be invariant throughout gestation in fetal liver and placenta, inasmuch as the difference in Crt values between gapdh and 18s rRNA (Δct = Ctr(gapdh) - Ctr(18s)) was constant (not shown). Moreover, the trends in gene expression that characterized ontogenesis in the placenta and fetal liver compared well in the relative (Figs. 1–3) and absolute quantification (Table 2) methods.

This profile of organic anion carriers in rat placenta qualitatively reflects what is known about human placenta (39, 47, 53, 54). However, interspecies differences emerge. In rodents, the endoplacental yolk sac is the major site of Mrp1 expression, but this has no structural equivalent in humans after the first trimester (11). Rather, the fetal endothelial capillaries of placental villi prominently express Mrp1 in humans (39, 54), but the fetal capillaries of the labyrinth zone of rat placenta do not. However, low expression of Mrp1 appeared on the basal side of the syncytiotrophoblast in the rat labyrinth zone, which agrees with the basal syncytiotrophoblastic localization of Mrp1 in human placental villi (39). Quantitative differences were also noted. The Mrp2 protein was immunolocalized to the apical syncytiotrophoblast membrane in human placental villi (54) but was undetectable when rat placentas were examined in the same way. Such discrepancies must be acknowledged when extrapolating data on fetal-maternal exchange of certain solutes from the rodent model to humans.

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ORGANIC ANION TRANSPORTERS IN RAT PLACENTA


