Asynchronous expression and colocalization of Bsep and Mrp2 during development of rat liver

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Zinchuk, Vadim S., Teruhiko Okada, Kunihiro Akimarum, and Harumichi Seguchi. Asynchronous expression and colocalization of Bsep and Mrp2 during development of rat liver. Am J Physiol Gastrointest Liver Physiol 282: G540–G548, 2002.—In the liver, function of the bile salt export pump (Bsep), a major canalicular exporter of bile salts, is complemented by activity of the multidrug resistance protein 2 (Mrp2), a canalicular organic anions transporter. Mrp2 was found capable of transporting various anticanancer drugs out of cells, eventually undermining their therapeutic potential and contributing to multidrug resistance. We employed a RT-PCR, immunohostaining, and immunofluorescence to examine their gene, protein expression, and distribution of antigenic sites in the rat liver during development from 16-day-old fetus to adult animal. Bsep mRNA was almost undetectable before birth. It was first clearly expressed in the liver of newborn rats. On the contrary, Mrp2 mRNA was seen before birth, although at low levels. In concert with mRNA expression, Bsep protein was undetectable before birth, while Mrp2 protein was already expressed. Both proteins were clearly detectable in the postnatal period. Confocal immunofluorescence microscopy showed asynchronous appearance of Bsep and Mrp2 proteins during development but their colocalization in the bile canaliculi once each one is expressed. During the gestational period, a weak immunofluorescence for Mrp2 was observed only in livers of 16-day-old embryos. No fluorescence for Bsep was seen. Both proteins were clearly visualizable after birth, although the pattern of immunostaining varied. These findings provide molecular evidence that expression of both Bsep and Mrp2 during development is transcriptionally regulated. They also point out the differences in relevance to the liver function of the systems responsible for canalicular transport of bile salts versus organic anions.

canalicular transporters; rat; ATP-binding cassette proteins; ontogenesis

FORMATION OF BILE is a unique function of the liver. Secretion of bile is an ATP-dependent process maintained by the function of specific transport ATPases (1, 12, 31, 39, 50). These independent canalicular transport systems belong to the superfamily of ATP-binding cassette (ABC) transporters and share common structural and functional characteristics (15, 29, 32, 48). ABC proteins are identified in various tissues and some of them were shown to be involved in multidrug resistance. They are also responsible for more restricted organ-specific functions depending on where they are expressed. In the liver, canalicular bile export pump (Bsep), also known as the sister of P-glycoprotein, is the major canalicular bile salt export pump of mammalian liver (7, 13). It was found that mutation of the gene encoding for a liver-specific ABC transporter leads to a progressive familial cholestasis type 2 (19, 42). Results were obtained demonstrating that Bsep plays an important role in the induction of intrahepatic cholestasis in mammalian liver (41). In addition, the canalicular isoform of the multidrug resistance-associated protein 2 (Mrp2), synonym to the canalicular multispecific organic anion transporter (cMOAT), is also confined to the canalicular domain and complements the function of Bsep by mediating export of amphiphatic anionic substrates (22, 28, 40, 46). In cultured cells, MRP2, a member of the MRP gene family, confers drug resistance by exporting cytotoxic drugs out of cells and therefore represents an important therapeutical target for development of inhibitors that can potentially interfere with this transport (4, 9). Both Bsep and Mrp2 have been recently cloned and sequenced (5, 13, 34).

Earlier studies established that specific hepatic functions are acquired at different stages of liver development. This process has been described as a precisely orchestrated series of molecular and morphogenetic events (11, 14, 16, 30). Mechanisms that govern Bsep and Mrp2 expression during development are of practical significance, because it was demonstrated that incomplete development of the bile salt transporting system by the end of the gestation period results in numerous pathological alterations (43). Immature function of hepatic excretion may cause a harmful accumulation of drugs in the plasma and increase susceptibility of the liver to cholestasis (21, 38). Recent investigations documented that cholestasis, either extra- or intrahepatic, reduces expression of Bsep and...
Mrp2 at the mRNA and protein levels (36, 45, 49). It is noteworthy that Mrp2 is affected by extrahepatic cholestasis significantly stronger than Bsep, attempting to suggest that stability of the expression of these canalicular transporters is different, and therefore mechanisms of regulation and physiological relevance to liver functions vary (26). A recent study examined hepatic bile salt and organic anion transporters in pregnant and postpartum rats and found their differential regulation (6). However, Bsep and Mrp2 were not characterized in the developing liver. Therefore, in the present study, we evaluated the gene and protein expression as well as in situ localization of Bsep and Mrp2 during development of rat liver from 16-day-old fetus to adult animal.

MATERIALS AND METHODS

Animals. Pregnant Sprague-Dawley rats with known mating dates were laparatomized at 16 and 20 days of gestation. Other experimental animals included newborn (23 days in utero), 1-wk-old, and adult rats. Newborn rats were killed after suckling. All procedures were approved by the Institutional Research Animal Care Committee.

Total RNA isolation. Total RNA was extracted using a Teflon homogenizer with the Isogen reagent (Nippon Gene, Tokyo, Japan) and quantified using ultraviolet absorbance at 260 nm. Integrity of samples was confirmed by gel electrophoresis.

Semiquantitative RT-PCR. Random primed synthesis of the first-strand cDNA from total RNA and PCR with specific primers was performed using a BcaBEST RNA PCR kit (Takara, Osaka, Japan). One milligram of total RNA used for RT reaction was then divided into five aliquots for PCR. For the purpose of quantitation, GAPDH was used as an internal control to normalize differences in cDNA loading. Specific primers were designed by analyzing the corresponding cDNA sequences (GenBank accession nos. U69487, L49379, and AF106860 for Bsep, Mrp2, and GAPDH, respectively) using the OLIQ0 primer analysis software. The primer sequences were as follows: 1) for Bsep (447–994): 5’ TGC TTA TGG GAG GCC TAG TAT 3’ (upper primer), 5’ GGG CTG ACA GCA AGA ATC 3’ (lower primer); 2) for Mrp2 (677–1472): 5’ GTC ACG GCC TCC TTT CTG 3’ (upper primer), 5’ AAC CCC AAC ACC TGG TAA 3’ (lower primer); 3) for GAPDH (942–1373): 5’ CAA CGA CCC CTT CAT TGA 3’ (upper primer), 5’ CAG TGA TGC CAT GCA CTG 3’ (lower primer). The amplified products for Bsep, Mrp2, and GAPDH were predicted to be 565, 813, and 449 bp in length, respectively. Preliminary experiments showed linear increase of the accumulation of PCR products at different amplification cycles (26 for Bsep and 24 for GAPDH), and therefore, PCR amplification was performed accordingly. It was carried out as follows: denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 1 min. Sequences of PCR products were confirmed in a separate experiment using the direct sequencing method (data not shown).

RT-PCR products were electrophoresed on 1.8% agarose gel and stained with ethidium bromide. DNA bands were visualized under ultraviolet light, and photoimage was recorded using an epifluorescence detector Epi-Light ultraviolet FA 1100 (Asin Cosmos R&D, Nagoya, Japan). Photoimages were scanned at 300 dpi and quantified using the NIH Image 1.6 software.

Western blotting. Approximately 50 mg of liver tissue was obtained from fetuses and rats of different age as described (13) with some modifications and proceeded similarly in all groups examined. The tissue was briefly washed free of blood and cut into small pieces in an ice-cold extraction buffer (250 mM/l sucrose, 95 mM/l NaCl, 45 mM/l Tris-HCl, pH 7.6), containing 0.025 trypsin inhibitor unit/ml of aprotonin and 0.1 mM/l phenylmethysulfonyl fluoride to eliminate the possible contribution of proteases (44). Samples were then homogenized in 10 vol of the buffer with a Polytron homogenizer (Kinematica, Lucerne, Switzerland) for 5 min. After filtration through the cheesecloth, the homogenate was centrifuged at 3,000 rpm for 30 min to remove cell debris. The pellet was discarded, and the collected supernatant was ultracentrifuged (model TL-100; Beckman Instruments, Palo Alto, CA) at 100,000 g for 1 h. The resulting pellet containing fractions of membrane proteins was recovered and suspended in a membrane suspension buffer (50 mM/l Tris-HCl buffer, pH 7.6, containing 50 mM/l HEPES, 0.2 mM/l d-glucoronic acid, and 250 mM/l sucrose). Concentration of proteins was measured with the DC protein assay method (Bio-Rad, Hercules, CA) using BSA as a standard. Proteins were aliquoted and stored at −70°C until further use. Equal amounts (~25 μg) of proteins were then boiled for 3 min in a

Fig. 1. Detection of bile salt export pump (Bsep) mRNA expression during development of rat liver from 16-day-old fetus to adult animal by RT-PCR analysis (A) and its corresponding relative density (B). Four independent experiments were performed, and a representative image is shown. Amplification cycles were 26 for Bsep and 24 for GAPDH. GAPDH mRNA was used as the standard to ensure equality of the loaded amount of cDNA. Bsep mRNA is almost undetectable during gestation but increases significantly right after birth. A: DNA ladder sizes in base pairs are given (left). Values of densitometry are expressed in relative units ± SD compared with adult rats as control.
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Fig. 2. Detection of multidrug resistance protein 2 (Mrp2) mRNA expression during development of rat liver from 16-day-old fetus to adult animal by RT-PCR analysis (A) and its corresponding relative density (B). Four independent experiments were performed and a representative image is shown. Amplification cycles were 26 for Mrp2 and 24 for GAPDH. GAPDH mRNA was used as the standard to ensure equality of the loaded amount of cDNA. Although weak, Mrp2 mRNA is expressed in fetuses. A: DNA ladder sizes in base pairs are given (left). Values of densitometry are expressed in relative units ± SD compared with adult rats as control.

sample buffer and separated through 10% SDS-PAGE (Bio-Rad) according to Laemmli (25) using a Mini-Protean II electrophoretic apparatus (Bio-Rad). Prestained molecular weight markers (Vector Laboratories, Burlingame, CA) were run in the same gels. Proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes by electroblotting. Ponceau S staining was performed to check for the equal protein transfer. Nonspecific binding was blocked by incubation in 5% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 Tris-base sodium (TTBS), blocked by incubation in 5% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 Tris-base sodium (TTBS), —

membranes were washed in TTBS, they were incubated with the primary polyclonal anti-Bsep and monoclonal anti-

Mrp2 antibodies, diluted 1:2,000, for another 1 h. Finally, the membranes were incubated with the biotin-horseradish peroxidase-streptavidin complex (Vector Laboratories), diluted 1:10,000, for 30–45 min at room temperature. After extensive washing with TTBS, membranes were exposed to the enhanced chemiluminescence-plus reagents (Amersham) according to the manufacturer’s protocol. Emitted light was documented on a Fuji RX-U X-ray film.

Immunofluorescent microscopy. Livers were excised, cut on small pieces, embedded in optimum cutting temperature compound (Miles, Elkhart, IN), and frozen in liquid nitrogen. After cutting with a cryostat, sections (6–8 μm thick) were picked up on poly-L-lysine-coated glass slides, air-dried, and exposed to acetone for 30 min at −20°C. Nonspecific binding was blocked with 10% goat serum in TBS for 30 min. The sections were then incubated with the mixture of rabbit anti-Bsep antibody (final dilution 1:100) and mouse anti-Mrp2 antibody (final dilution 1:50) for 1 h at room temperature. After rinsing with TBS, the sections were incubated with the mixture of corresponding secondary antibodies (conjugated with Alexa 488 and Alexa 594, respectively; Molecular Probes, Eugene, OR), diluted 1:400, for another 1 h at RT. After a final washing procedure, the sections were mounted with a ProLong antifade reagent (Molecular Probes, Leiden, The Netherlands), coverglassed and examined using confocal optics. In controls, specimens were either incubated with nonimmune IgG or the primary antibody was omitted from the labeling process.

Confocal laser scanning microscopy and imaging. Immunocytochemical staining was examined using a confocal laser scanning microscope LSM 410 (Carl Zeiss, Jena, Germany), which is capable for accurate visualization of fluorescent markers, because the thin optical section generated by the instrument eliminates the confounding effects of out-of-focus fluorescence (51). Images were captured on MO disk and

Fig. 3. Expression of Bsep (A) and Mrp2 (B) proteins during development of rat liver from 16-day-old fetus to adult animal detected by immunoblotting. Three independent experiments were performed and representative images are shown. Membrane fractions (25 μg of each protein) were subjected to SDS-PAGE electrophoresis and then transblotted to PVDF membranes as described in MATERIALS AND METHODS. Protein blots, probed with the anti-Bsep and anti-Mrp2 antibodies, reveal bands at 160 and 190 kDa, respectively. Bsep protein is not expressed before birth. Mrp2 protein is expressed both before and after birth. Positions of molecular mass markers (in kDa) are indicated on the left.

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stored for further editing and enhancement. Digital images were processed on a Macintosh Dual G4 Power PC (Apple Computer, Cupertino, CA) and composed with Adobe Photoshop 6.0.1 software (Adobe Systems, San Jose, CA). Images, with contrast and brightness adjusted to optimally represent the original pattern of immunocytochemical staining, were printed out directly using a Fuji Pictrography 3000 printer (Fuji Photo Film, Tokyo, Japan).

RESULTS

During pre- and postnatal development, liver acquires its functions via a precisely regulated chain of morphogenetic events. To find out when, how, and where Bsep and Mrp2 genes and proteins are expressed in the developing rat liver, we employed semi-quantitative RT-PCR, immunoblotting, and confocal immunofluorescent microscopy.

Detection of Bsep and Mrp2 mRNA. Bsep mRNA was practically undetectable in the liver of 16-day-old fetuses. It was first clearly detected in the liver of newborn rats (Fig. 1A). Thereafter, mRNA expression levels underwent a steady and gradual increase. In adult animals, its levels increased approximately three times compared with the expression seen in newborn rats. Expression of Mrp2 mRNA showed a different profile of the developmental increase (Fig. 2A). The first signs of Mrp2 mRNA were detected in the liver of 16-day-old fetuses, although level of expression was very low. The level of expression almost doubled in the liver of 20-day-old fetuses, but still accounted only for ~10% of the level in adults (Fig. 2B). Liver of newborn rats revealed more than triple increase of the level of Bsep mRNA expression compared with 20-day-old fetuses (Fig. 1B). At birth, levels of expression of both Bsep and Mrp2 constituted approximately one-third of levels detected in adult animals. One-week-old pups showed further increase of the expression. In the liver of adult rats, levels of Mrp2 mRNA expression were considerably higher.

Immunoblot detection of Bsep and Mrp2 protein. Bsep protein was not expressed in fetal livers. With the birth of animals, a band of the weight of ~160 kDa has become detectable (Fig. 3A). Expression of Bsep protein underwent a very significant increase. On the contrary, expression of Mrp2 protein (band of the weight of ~190 kDa) was detected in livers of 16- and 20-day-old fetuses and tended to increase gradually after birth (Fig. 3B).

Immunofluorescent microscopy of Bsep and Mrp2. Liver of 16-day-old fetuses consisted mainly of hematopoietic stem cells and mitotically active hepatocytes. Hepatic cell cords were seldom seen. No fluorescence for Bsep was detected (Fig. 4A), whereas
weak staining for Mrp2 was already noticed (Fig. 4, B and C). On day 20 of gestation, the number of hematopoietic cells decreased and embryonic liver revealed hepatic cell cords and better-defined canaliculi spaces. In more than one-half of preparations, antibodies for Bsep and Mrp2 stained what appeared to be dilated bile canaliculi (Fig. 4, D and F). The staining was coarse and extended to subapical portions of hepatocytes. Fluorescence for Mrp2 was more pronounced (Fig. 4E). At birth, well-formed bile canaliculi displayed staining for both Bsep and Mrp2 (Fig. 5, A–C). Anti-Bsep and anti-Mrp2 antibody revealed the classic branching pattern of canaliculi. The antigens appeared colocalized, however, intensity of fluorescence was stronger for Bsep than for Mrp2. Although Bsep and Mrp2 remained confined to the bile canalicular system, the staining pattern of canaliculi in the newborn and adult rats was noticeably different. In the newborn animals, the staining was seen as rather fuzzy (Fig. 5, A–C), whereas in adults it was very compact and sharply defined the canaliculi (Fig. 5, G–I). In livers of 1-wk-old rats, fluorescence was frequently seen in subapical areas of hepatocytes likely belonging to the so-called subapical compartment (Fig. 5, D–F). Only occasionally similar areas showed fluorescence in adult animals. Sinusoidal and basolateral domains of the plasma membrane of hepatocytes as well as their cytoplasm.
were left unstained in all preparations. Lobular gradients of staining for either Bsep or Mrp2 were not noticed.

Tables 1 and 2 briefly summarize results described above.

**DISCUSSION**

The goal of the present study was to obtain new information regarding the expression of Bsep and Mrp2, two independent canalicular transporters of mammalian liver, by following them during pre- and postnatal liver development. We examined gene expression of Bsep in the liver development earlier than Bsep. We detected that Mrp2 protein is expressed earlier as well. We also observed that, once expressed, Bsep and Mrp2 proteins share apical domain of the hepatocyte plasma membrane as their only in situ location in the liver, either in the pre- or postnatal period. What is of significance in these findings that can provide clues for better understanding of the function of these canalicular transporters?

Gene expression of Bsep, an ATP-dependent canalicular transporter of bile salt into bile, was first clearly detected in the liver of newborn rats, although a weak expression was seen in the liver of 20-day-old fetuses. After birth, levels of expression of mRNA showed gradual increase with its maximum detected in livers of adult animals. It should be mentioned that earlier studies reported that taurocholate, the predominant bile salt in the bile of adult rats, becomes detectable on day 10 of gestation. However, this is likely due to the occurrence of a mother-fetus transfer, because at this time the liver of the fetus is still in primordial state. Then, the size of the pool of bile salts continues to increase, although the rate of increase slows and concentration of bile salts at birth is yet to reach the levels of adulthood (27). After birth, secretion and reabsorption of bile salts in suckling rats continue to be reduced compared with adult animals (43, 52). The clear correlation between the Bsep gene expression and the time of birth when hepatocytes reveal all structural features of fully differentiated cells suggests that the molecular basis for Bsep function is already formed by this time. These results are in agreement with the report showing that expression of Bsep depends on cell differentiation (33) and supported by the current view that Bsep is a dominant ATP-dependent bile salt export pump of mammalian liver (13, 41).

Gene expression of Mrp2, another canalicular transporter that mediates ATP-dependent transport of organic anions (e.g., bilirubin diglucuronide) into bile and contributes to the bile salt-independent bile flow, was different from that of Bsep. Mrp2 mRNA was first detected in the liver of 16-day-old fetuses, although the expression level was low. The level of expression doubled in livers of 20-day-old fetuses and then tripled in livers of newborn rats. Mrp2 mRNA expression did not appear to be stimulated by suckling as profoundly as Bsep. It is known that Mrp2 is defective in Eisai hyperbilirubinemic and TR− mutant rats (18, 34). In humans, patients with mutations of the MRP2 gene develop the Dubin-Johnson syndrome, an autosomal recessive liver disorder characterized by chronic hyperbilirubinemia (34, 47). Using cell culture, it was demonstrated that MRP2 is related to drug resistance, because overexpression of MRp2 results in resistance to carcinostatics (9, 17, 20, 23). MRp2 was also detected in various carcinomas (22). Detectability of Mrp2 mRNA earlier in the liver development, taken together with the observation that suckling does not stimulate its expression as much as it stimulates the expression of Bsep mRNA, may indicate that suckling exerts different effects toward ATP-dependent canalicular transport of organic anions versus bile salts.

Expression of Bsep and Mrp2 proteins during development was in general agreement with the expression of their mRNA. Bsep protein was clearly expressed only at the time of birth, whereas Mrp2 protein was identifiable before and after birth, thus indicating transcriptional regulation of both transporters during liver development. Considerable increase of the expression of Bsep mRNA and protein and Mrp2 mRNA between the 20-day-old fetus and a newborn demonstrates that the presence of these canalicular transporters rapidly increases during this period of time as the organ readies for its function after birth. It is worth mentioning that expression of Bsep mRNA and protein showed dissociation between the groups of newborn rats and 1-wk-old pups, tempting to assume that there

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might also be an involvement of additional mechanisms of posttranscriptional regulation. Further studies are required to clarify this issue.

Immunostaining for Bsep and Mrp2 proteins showed both antigens confined to the canalicular domain of the hepatocyte plasma membrane. It was noticed that intensity of Mrp2 immunofluorescence during development increased greater than its protein expression on Western blots. This may be due to the different sensitivity of these techniques. Both proteins were colocalized throughout all period of development, suggesting their affinity. This observation is in concert with the results of other investigators on the in situ localization of Bsep and Mrp2 and agrees with the study on polarization of the hepatocyte cell surface from the earliest time at which the liver is identifiable in the fetus (11, 13, 20). A very recent report of Akita et al. (2) gave further support to these findings by demonstrating coexpression of Bsep and Mrp2 in canalicular membrane vesicles. Importantly, immunochemical data showed that Bsep protein is not expressed in the liver of 16-day-old fetuses. Although weakly, both Bsep and Mrp2 are detectable in the liver on day 20 of embryonic development. At this time, Mrp2 protein is seen expressed stronger than Bsep. On the contrary, expression of Bsep is stronger than Mrp2 in the postnatal period. These observations may be explained by the fact that molecular composition of the plasma membrane domains in the fetal hepatocyte differs from that in the adult (11). It is also known that even in a late stage of fetal development, rat liver is yet to reach its fully differentiated state (16). Remarkably, changes of the developmental expression of Bsep and Mrp2 proteins observed here were consistent with the timing of structural maturation of the bile canaliculi and their secretory system (8). Changing patterns of immunochemical localization of Bsep and Mrp2 proteins before and after birth supported the view that the full functional maturation of the bile synthesis and secretion is not occurring until after birth (10, 27).

Interestingly, at no period of liver development, could immunostaining be detected beyond the canalicular system. One might have envisioned a scenario according to which the plasma membrane proteins, whatever their domain in the hepatocyte of adult rats, are distributed in fetal hepatocytes in a random fashion, mature, and eventually move toward their designated location together with the organ’s ontogenesis. Such an assumption might have been justified by the well-developed dynamic properties of the hepatocyte plasma membrane proteins: delivery of canalicular proteins to the basolateral domain and then back to the apical domain, for instance, are necessary steps in hepatocyte biogenesis (3). However, from its earliest appearance, immunofluorescence for both Bsep and Mrp2 was detectable exclusively in the apical domain. This finding indicates that, whereas structural and functional maturation of fetal hepatocytes is far from being completed, they already reveal precise canalicular polarization of Bsep and Mrp2 proteins.

In addition to the canalicular membrane, we have anticipated finding fluorescence for either Bsep or Mrp2 intracellularly as well. Signs of Bsep protein were previously detected in the liver of adult rats within the so-called subapical compartment (13). The latter is located in a close proximity to the apical domain of hepatocytes and connects the transcytotic and the apical-to-lysosomal pathways (37). It is also known that such pathological conditions like hyperosmotic stress and cholestasis cause partial redistribution of Mrp2 into subapically located intracellular vesicles (24, 36, 45). As expected, we observed immunofluorescence for Bsep or Mrp2 in areas of the cytoplasm of hepatocytes adjacent to the apical domain where subapical compartment is supposed to be situated, although very seldom in adult rats. The staining was strongest and most readily detectable in the liver of newborn rats and especially 1-wk-old pups. These observations are likely reflecting the different amount of proteins at their final, canalicular localization and/or the need for them to be supplied continuously and to ensure the functional maturation of the canaliculi, because it is known that processes of secretion and reabsorption of bile salts in suckling rats are still functionally incomplete (40, 52). They also imply that subapical compartment, an important crossing of incoming and outgoing messages in the hepatocyte, may be involved in the process of delivery of canalicular transporters. Remarkably, a recent study of Paulusma et al. (36) reported that even when Bsep and Mrp2 undergo internalization toward the intracellular compartment in response to bile duct ligation, they still maintain their colocalization and remain to be active.

In conclusion, the study revealed asynchronous expression and colocalization of Bsep and Mrp2 during pre- and postnatal liver development. Although further studies are needed, our results show that expression of Bsep and Mrp2 during development is likely regulated at the transcriptional level as well as imply that these complementary functioning canalicular transporters are of different physiological significance for the mammalian liver.

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