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Phylogenic and ontogenic expression of hepatocellular bile acid transport

(liver/organic anion transport/phylogeny/ontogeny)

J. L. BOYER*†‡, B. HAGENBUCH§, M. ANANTHANARAYANAN†, F. SUCHY†, B. STIEGER§, AND P. J. MEIER§

*Mt. Desert Island Biological Laboratory, Salsbury Cove, ME 04672; †Liver Center and Department of Medicine and Pediatrics, Yale University School of Medicine, New Haven, CT 06510; and §Division of Clinical Pharmacology and Toxicology, University Hospital, Zurich CH-8091, Switzerland

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ABSTRACT **The phylogenic and ontogenic expression of mRNA for the Na^+/bile acid cotransporter was determined by Northern analysis utilizing a full-length cDNA probe recently cloned from rat liver. mRNA was detected in several mammalian species, including rat, mouse, and man, but could not be found in livers from nonmammalian species, including chicken, turtle, frog, and small skate. When expression of the bile acid transporter in developing rat liver was studied, mRNA was detected between 18 and 21 days of gestation, at the time when Na^+-dependent bile acid transport is first detected. Two hepatoma cell lines (HTC and HepG2), the latter of which is known to have lost the Na^+/bile acid cotransport system, also did not express mRNA for this transporter. Finally, when mRNA from the lower vertebrate (the small skate) was injected into *Xenopus* oocytes, only a sodium-independent, chloride-dependent transport system for bile acids was expressed, confirming the integrity of the mRNA and consistent with prior functional studies of bile acid transport in this species. These findings establish that the Na^+/bile acid cotransport mRNA is first transcribed in mammalian species, a process that is recapitulated late during mammalian fetal development in rat liver, and that this mRNA is lost in dedifferentiated hepatocytes. In contrast, the mRNA for a multispecific Na^+/independent organic anion transport system is transcribed earlier in vertebrate evolution.**

The hepatic uptake of organic anions is a fundamental property of the liver of all vertebrates. The transport mechanisms that carry out this function are responsible for the plasma clearance of endogenous substances such as bilirubin and bile acids and other naturally occurring substances as well as many exogenous compounds and drugs (1). At least two separate transport systems have been identified in mammalian liver by means of conventional transport assays using isolated cells (2, 3), perfused livers (3, 4), and isolated membrane vesicles (5–8). One system derives its energy from the inwardly directed sodium gradient (a secondary active transport mechanism), whereas the other is facilitated by chloride anions (3, 9). Bile acids (specifically, taurocholate) are transported from portal blood into hepatocytes preferentially by the sodium-dependent system (5–7), whereas bilirubin and a prototype substrate, sulfobromophthalein (BSP), utilize sodium-independent mechanisms (9). Phylogenic studies of taurocholate transport suggest that the sodium-dependent transporter is absent in hepatocytes from lower vertebrates (cartilaginous fishes) (10, 11), whereas studies in liver plasma membrane vesicles from the developing fetus of the rat indicate that expression of this transporter is first detected at 20 days of gestation in this mammalian species (12). Sodium-dependent transporters for bile acid uptake in liver have been identified functionally in a number of mammalian species, including man (13).

Recently, a sodium-dependent bile acid transporter from adult rat liver has been expressed in *Xenopus laevis* oocytes (14) and a full-length sequence for the cDNA has been determined using this expression system (15). The availability of this clone now allows a more definitive examination of the phylogenic and ontogenic expression of this transporter at the mRNA level.

MATERIALS AND METHODS

Animals. Mature *X. laevis* females were purchased from H. Kahler (Hamburg, F.R.G.) and maintained under standard conditions as described (14). Adult Sprague–Dawley rats (250 g) were obtained from the Süddeutsches Tierzuchtinstitut (Tutlingen, F.R.G.); gestational and weanling Sprague–Dawley rats were obtained from Charles River Breeding Laboratories. BALB/c mice were purchased from the Institut für Versuchstierkunde, University of Zurich; chicken and turtle were obtained from local sources. Small skates (*Raja erinacea*) were obtained by trawl from the waters off Southwest Harbor, ME, and maintained in salt water tanks with flowing sea water at the Mt. Desert Island Biological Laboratory, Salsbury Cove, ME.

Source and Culture Conditions of Hepatocytes, HTC Cells, and HepG2 Cells. Hepatocytes were isolated by collagenase perfusion and obtained together with HTC cells from the Yale Liver Center Core Facility. HepG2 cells were grown as monolayers in T-25 flasks in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, glutamine and penicillin (100 units/ml), and streptomycin (100 μg/ml). HTC cells were grown in DMEM/Ham's F-12 mixture containing the same supplements as described for HepG2 cells. Fresh medium was added twice a week and cells were passaged once a week after trypsinization with trypsin/EDTA (Sigma). RNA was prepared from these cells after 5–15 passages as described (16).

Isolation of RNA. Total RNA was prepared either by a single-step guanidinium thiocyanate/phenol/chloroform extraction procedure (16) or by cesium chloride gradient according to published methods (17). mRNA was purified by oligo(dT)-cellulose chromatography (18).

Northern Blotting. Samples of total or mRNA were separated by electrophoresis on agarose/formaldehyde gels, transferred to a nylon membrane [Hybond N (Amersham) or GeneScreen*Plus* (New England Nuclear)], and UV crosslinked. Blots were hybridized at 42°C overnight at 1–2 × 10^6 cpm/ml in standard hybridization solution containing 50% formamide and 100–200 μg of salmon sperm DNA per

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Abbreviation: BSP, sulfobromophthalein.

‡To whom reprint requests should be addressed at: 1080 Laboratory of Medicine and Pediatrics, Department of Medicine, Box 3333, Yale University School of Medicine, New Haven, CT 06510.

ml. Stringency was controlled using different salt concentrations and temperatures in final wash solutions. cDNA probes were labeled using a random-primed labeling kit (Boehringer Mannheim) to a specific activity of 10^8–10^9 cpm/μg.

Expression of mRNA in *Xenopus* Oocytes. Oocytes were prepared and maintained in culture as described (14). Four to 5 days after injection of 50 nl of mRNA (1 mg/ml) prepared from skate liver, the uptake of [^{3}H(G)]taurocholic acid (2.1 Ci/mmol; 1 Ci = 37 GBq; DuPont/New England Nuclear), [^{35}S]BSP (4 Ci/mmol) prepared according to Kurisu *et al.* (19) and kindly provided by Allan Wolkoff (Albert Einstein School of Medicine), or [^{3}H]taurine (25.6 Ci/mmol) was determined as described (14, 20–22).

RESULTS

To determine the phylogenic expression of the cloned mRNA for the rat liver Na^+/bile acid cotransporter, a ^{32}P-labeled cDNA probe (926 nucleotides) was prepared and hybridized with mRNA prepared from livers of the small skate, frog, turtle, chicken, and several mammalian species, including rat, mouse, and man. As illustrated in Fig. 1, the probe failed to hybridize using low stringency washing conditions with mRNA from nonmammalian livers. In contrast, all mammalian liver mRNA samples gave a positive hybridization signal, indicating that they all contain mRNA homologous to the cDNA encoding the rat liver sodium-dependent bile acid transport system. Thus, the hepatocellular Na^+-dependent bile acid uptake system is selectively expressed in mammalian liver. The weaker signal with human liver mRNA (Fig. 1) reflects either a lower degree of homology or a lower abundance of the corresponding mRNA as compared to rat and mouse liver.

Since species distribution (evolution) of a protein is generally recapitulated in its ontogenesis, we next studied the expression of the Na^+/bile acid cotransporter mRNA in developing rat liver. As illustrated in Fig. 2, a faint positive

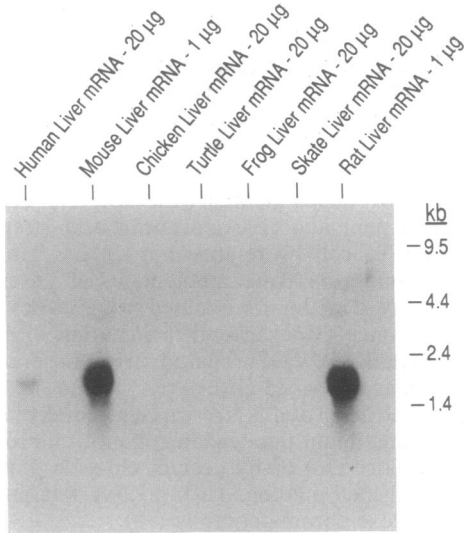

FIG. 1. Northern blot hybridization of mRNA from representative vertebrate species. The RNA samples were separated on 1% agarose/formaldehyde gels, transferred to Hybond nylon membranes, and hybridized after crosslinking with UV light with an *Eco*RI fragment (nucleotides 261–1187) of the cloned Na^+/bile acid cotransporter from rat liver, previously labeled with ^{32}P by random priming. Low stringency washing conditions consisted of twice, 15 min each, at room temperature with 2× SSC/0.1% SDS (1× SSC = 0.15 M NaCl/15 mM sodium citrate) and once, 15 min, at 59°C with 1× SSC/0.1% SDS. Blots were prehybridized for 6 hr, hybridized at 42°C with 50% formamide for 18 hr, and exposed for 24 hr. kb, Kilobases.

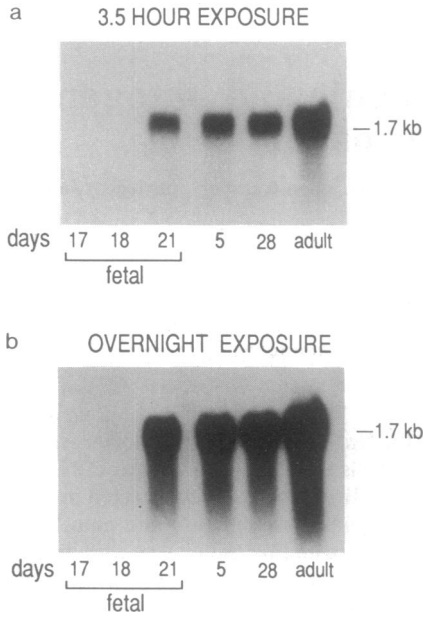

FIG. 2. Northern blot hybridization of mRNA (5 μg) prepared from rat liver of the 17-, 18-, and 21-day gestating rat and 5- and 28-day neonatal and adult rat using the ^{32}P-labeled bile acid cDNA probe. After separation of the RNAs on 1.2% agarose gels, the RNA was transferred to GeneScreen*Plus* nylon membranes, crosslinked by UV light, and hybridized with the cDNA probe using the following washing conditions: twice, 15 min each, at room temperature with 2× SSC; once, 30 min, at 60°C with 2× SSC/1.0% SDS; twice, 30 min each, at room temperature with 0.1× SSC. Blots were exposed for 3.5 hr or overnight for 16 hr.

hybridization signal was first detected during the prenatal period at day 18 after prolonged (overnight) exposure of the autoradiograms. However, a strong hybridization signal was detected at day 21, which corresponded approximately to the time when sodium-dependent bile acid transport can be first demonstrated in basolateral membrane vesicle preparations isolated from fetal rat liver (12). Thereafter, the mRNA levels increased progressively and gradually approached adult levels in the postnatal period (Fig. 2). To quantitate message levels during development, the Northern blot shown in Fig. 2 was exposed for 0.5, 1.0, and 1.75 hr and the autoradiograms were scanned using a laser densitometer. The resulting absorbance values were linear with increasing exposure time, suggesting that the autoradiographic signal was linear to the amount of message in the sample. By assigning an arbitrary value of 1.0 to the quantity of message on fetal day 21, it was observed that the mRNA levels increased 1.9-, 2.4-, and 4.7-fold on postnatal days 5 and 28 and adult livers, respectively. The blot shown represents a typical experiment and a similar pattern of increase in message levels was seen in other experiments. The fetal mRNA samples were also probed with a cDNA for human fibroblast γ-actin, which showed a positive signal, suggesting that the delayed transcription is specific for the bile acid transporter message (data not shown). This late ontogenic expression of the Na^+/bile acid cotransporter system correlates with the evolutionary expression pattern demonstrated in Fig. 1.

To determine whether the Na^+/bile acid cotransporter message is expressed in dedifferentiated hepatocytes, mRNAs from two hepatoma cell lines (HTC and HepG2) were also probed by Northern analysis. As seen in Fig. 3, expression of this mRNA was completely absent in these rat and human hepatoma cell lines, respectively. The blots were stripped and reprobed with γ-actin, indicating that the absence of expression of the bile acid transporter message is not

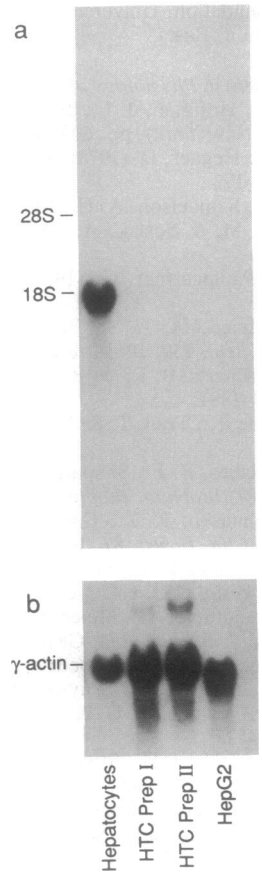

FIG. 3. (*a*) Northern blot hybridization of mRNAs from hepatocytes and HTC and HepG2 cells. Oligo(dT)-purified mRNAs (25–30 μg per lane) were fractionated on 1.2% formaldehyde/agarose gels, blotted to nylon membranes (GeneScreen*Plus*), and probed with full-length cDNA encoding Na^+/bile acid cotransporter. The blots were washed twice to a final stringency of 0.1× SSC at room temperature, 30 min each, and exposed to x-ray films for 4 hr at −70°C. (*b*) Northern analysis of the same blot as in *a* reprobed with γ-actin cDNA.

due to a reduced amount or degradation of RNA applied to the blots.

To confirm the absence of the mRNA for the mammalian liver Na^+/bile acid cotransporter in lower vertebrates on a functional level, we next injected the mRNA prepared from livers of the small skate into oocytes. As demonstrated in Fig. 4, no Na^+-dependent taurocholate transport activity could be observed. However, Na^+-independent taurocholate uptake was significantly higher in mRNA-injected oocytes when compared to noninjected controls. In addition (Fig. 4), the expressed Na^+-independent taurocholate uptake was dependent on the presence of extracellular chloride.

A similar Na^+-independent chloride dependency was also observed for the uptake of the organic anion BSP, as described in rat hepatocytes and functionally expressed in oocytes after injection of rat liver mRNA (22).

Finally, to more fully assess the functional competence of the mRNA, the Na^+ dependence of [^{3}H]taurine uptake was also determined in the oocyte system. In contrast to taurocholate or BSP, the uptake of taurine was stimulated by extracellular Na^+ (Fig. 4), as observed in isolated skate hepatocytes (23).

DISCUSSION

Recently, a full-length cDNA for the Na^+/bile acid cotransport system in rat liver has been cloned that mediates the

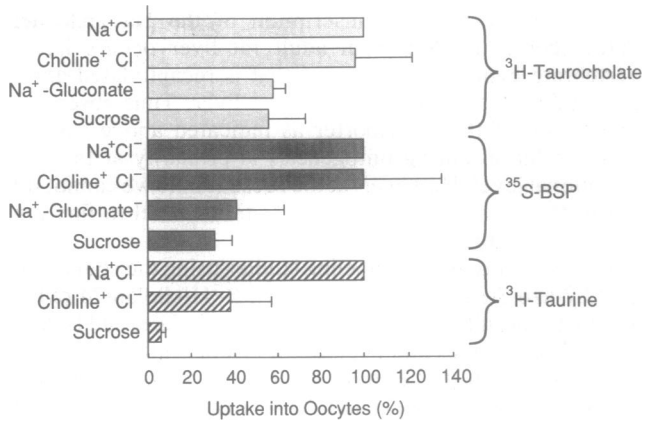

FIG. 4. Effects of extracellular ion substitution on 17 μM [^{3}H]taurocholate, 2–4 μM [^{35}S]BSP, and 0.76 μM [^{3}H]taurine uptake in *Xenopus* oocytes injected with 50 ng of skate liver mRNA (injection volume, 50 nl). Bovine serum albumin (BSA, 0.05%) was included in the incubation medium when [^{35}S]BSP uptake was used as described (22). After 3–4 days of incubation, uptake of the respective ligand was measured for 3–4 hr at 20°C in the presence of 100 mM NaCl, choline chloride, sodium gluconate, or 200 mM sucrose. Cold stop solutions of similar composition containing appropriate ligand [1 mM taurocholate, 0.74 mM BSA (5%), or 10 mM taurine] were used to reduce nonspecific binding. Each bar represents the mean ± SD of up to 40 uptakes in two to four separate oocyte isolations. The data are normalized to control (NaCl) values of 100% for each experiment.

hepatic uptake of this bile acid. This cloned transporter is strictly sodium-dependent and codes for a glycosylated protein of 362 amino acids with a calculated molecular mass of 39 kDa (15). Previous Northern blot analysis with the cloned probe indicated crossreactivity with mRNA from several mammalian species, including mouse, guinea pig, rabbit, and man, as well as mRNA from rat kidney and ileum, tissues that are also involved in bile acid transport (15).

In the present study we have utilized this cDNA probe to study the phylogenic and ontogenic expression of the mRNA for this transporter. Under low stringency conditions, no homologous mRNA can be found in any of the livers from nonmammalian species. The absence of mRNA for this transporter in nonmammalian liver is in keeping with previously published functional studies in isolated hepatocytes from marine elasmobranchs that demonstrate uptake of taurocholate into skate liver only by sodium-independent transport mechanisms (11, 24). The absence of a functional Na^+-dependent bile acid transporter in this lower vertebrate species was further confirmed by expressing this transport system in oocytes following injection of skate liver mRNA. Taurocholate transport into these oocytes occurred only by sodium-independent mechanisms. It is of interest that the uptake of taurocholate was also chloride dependent, similar to the transport of BSP. A high-affinity, chloride-dependent transport system for BSP has been demonstrated previously in rat hepatocyte cultures and recently expressed in *Xenopus* oocytes (22). Preliminary oocyte expression experiments indicate that the kinetic properties and substrate specificity for the BSP transport system in skate and rat liver are similar. This suggests that the multispecific organic anion transporter evolved early in vertebrate evolution and retained its functional characteristics in mammalian liver.

The present ontogenic studies indicate that the mRNA for the rat Na^+-dependent transporter is absent through most of gestation and is first detected toward the end of gestation on fetal days 18–21. These findings provide definitive support for conventional transport studies that first demonstrated functional evidence for Na^+-dependent transport in basolateral membrane vesicles prepared from the fetal rat liver just prior to birth (12). Together these phylogenic and ontogenic

studies establish that transcription of the Na^+/bile acid cotransporter mRNA from adult rat liver occurs late in vertebrate evolution, a process that is recapitulated during mammalian fetal development in rat liver. Thus absence of expression of this transporter as indicated above suggests that regulation during ontogenesis is primarily at the transcriptional level. However, at the present time we cannot rule out further control at post-transcriptional level to fine tune the carrier expression.

Several photoaffinity labeling studies using the sodium salt of {7,7-azo-3α,12α-dihydroxy-5β-([3β-^{3}H]cholan-24-oyl)-2-aminoethanesulfonic acid} (7,7-azotaurocholate) and isolated plasma membranes and hepatocytes from rat liver as well as protein isolation studies have identified two distinct proteins of approximately 54 kDa and 48–49 kDa (25–27). A 55-kDa organic anion-binding protein has also been identified in rat liver cell sinusoidal plasma membrane subfractions using photoactivated [^{35}S]BSP (28). When liver plasma membranes from lower vertebrates (small skate) are photolabeled with 7,7-azotaurocholate, only a 54- to 55-kDa protein is labeled and Scatchard analysis reveals only one binding site (11). These studies are in contrast to the studies in the rat where two proteins are labeled and two binding sites are detected by Scatchard plots (11). Thus it seems likely that there are two transporters for the hepatic uptake of organic anions in mammalian species but that transport of these ligands in lower vertebrates occurs only by the multispecific sodium-independent transporter(s) that is facilitated by chloride ions. The Na^+/bile acid cotransport system presumably evolved in mammalian species when the need for a more rapid and efficient transporter for bile acids developed, perhaps as a consequence of the greater turnover rates of bile acids in the enterohepatic circulation of these species.

Whether or not this transporter is represented by the 48- to 49-kDa bile acid photolabeled protein is not yet entirely clear since *in vitro* translation of the cDNA for the rat liver Na^+/bile acid cotransporter with dog pancreatic microsomes revealed a protein of only 39 kDa (15), although it is possible that the protein is not fully glycosylated in this *in vitro* system. Antibodies to an adult rat liver basolateral membrane 48-kDa protein detect a 48-kDa protein in gestational rat liver by immunoblot at the same time that sodium-dependent bile acid transport is first detected (29). Bile acid photoprobes fail to detect a 48- to 50-kDa protein in neonatal liver (30) and in HepG2 cells that have lost their sodium-dependent transport system as assessed by functional transport assays (27). Northern analysis of HTC and HepG2 cell mRNAs also fails to hybridize with the Na^+-dependent bile acid probe from rat liver, confirming that these hepatoma cell lines do not express the mRNA for this transporter and have lost this differentiated function of the adult mammalian hepatocyte. It remains to be determined whether the cloned Na^+/bile acid cotransporter and the 48- to 49-kDa basolateral protein are the same or different gene products.

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