Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 10629–10633, December 1991
Medical Sciences

Functional expression cloning and characterization of the hepatocyte Na^+/bile acid cotransport system

(liver/organic anion transport)

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Communicated by Rudi Schmid, August 30, 1991

ABSTRACT **Liver parenchymal cells continuously extract high amounts of bile acids from portal blood plasma. This uptake process is mediated by a Na^+/bile acid cotransport system. A cDNA encoding the rat liver bile acid uptake system has been isolated by expression cloning in *Xenopus laevis* oocytes. The cloned transporter is strictly sodium-dependent and can be inhibited by various non-bile-acid organic compounds. Sequence analysis of the cDNA revealed an open reading frame of 1086 nucleotides coding for a protein of 362 amino acids (calculated molecular mass 39 kDa) with five possible N-linked glycosylation sites and seven putative transmembrane domains. Translation experiments *in vitro* and in oocytes indicate that the transporter is indeed glycosylated and that its polypeptide backbone has an apparent molecular mass of 33–35 kDa. Northern blot analysis with the cloned probe revealed crossreactivity with mRNA species from rat kidney and intestine as well as from liver tissues of mouse, guinea pig, rabbit, and man.**

Bile formation is a major function of the liver in all vertebrate animal species and its correct interplay with other metabolic tasks of the liver has considerable significance for overall body economy. For example, bile represents the major route of cholesterol excretion from the body. In addition, bile provides an important excretory pathway for various endogenous (e.g., bile salts and bilirubin) and exogenous (e.g., many drugs and drug metabolites) organic compounds that cannot be easily eliminated from the body by the kidneys. To generate ongoing bile flow, hepatocytes must continuously transport bile acids from sinusoidal blood plasma into bile canaliculi against a steep concentration gradient. The first step in this overall vectorial transport process is mediated by a secondary active, Na^+ gradient-driven bile acid uptake system at the basolateral (sinusoidal and lateral) plasma membrane domain of hepatocytes (1, 2). This Na^+/bile acid cotransport system has been well characterized in a number of experimental systems (e.g., perfused rat liver, isolated hepatocytes, plasma membrane vesicles) with respect to its driving force, its transport kinetics, and its substrate specificity (3, 4). Furthermore, a 48- to 50-kDa glycoprotein has been identified as the putative transport protein (5–7). However, the exact molecular structure of this important hepatocellular bile acid transport system has not yet been determined nor has it been characterized on the mRNA and cDNA levels.

We have recently demonstrated that injection of rat liver poly$(A)^+$ RNA into *Xenopus laevis* oocytes resulted in the functional expression of the basolateral Na^+/bile acid cotransporter of hepatocytes (8). Furthermore, a 1.5- to 3.0-kilobase (kb) size class of mRNA was sufficient to generate the Na^+-dependent bile acid (taurocholate) uptake signal. In this study, we report the successful expression cloning and characterization of a cDNA encoding the rat liver basolateral Na^+/taurocholate cotransporting polypeptide.§

MATERIAL AND METHODS

Animals. Mature *X. laevis* females were purchased from H. Kähler (Hamburg, F.R.G.) and kept under standard conditions as described (9).

Construction of a cDNA Library. Rat liver mRNA was prepared and size-fractionated as described (8). cDNA was synthesized from the active mRNA size class using the cDNA synthesis plus kit (Amersham) and the oligo(dT) primer. After addition of *Bst*XI linkers, the cDNA was inserted into the *Bst*XI sites of Bluescript KS(+) (Stratagene). To avoid self-ligation of nonrecombinant plasmids, the polycloning site of the vector was modified (H.L., unpublished data). Recombinant plasmids were introduced into *Escherichia coli* MC1061 by electroporation (Gene Pulser, Bio-Rad). Starting with 5 μg of mRNA, 1.5×10^7 colonies with insert sizes between 1.5 and 2.3 kb were obtained.

Screening of the cDNA Library. Colonies (2.7×10^6) of the library were screened by injecting *in vitro* synthesized mRNA into oocytes and measuring Na^+-dependent taurocholate uptake (8). Initially mRNA was prepared from pools of 500–250,000 clones. Once a positive pool had been identified, it was further subdivided and screened (10) until a single positive clone [called prLNaBA (an isolated cDNA clone encoding the rat liver Na^+/bile acid cotransport system)] was isolated. For *in vitro* synthesis of mRNA, plasmids were isolated using the Qiagen plasmid kit (DIAGEN GmbH, Düsseldorf, F.R.G.) and cut with *Pvu* I. Capped mRNA was synthesized using T3 or T7 RNA polymerase (Promega) in the presence of the capping analogue $m^7G(5')ppp(5')G$ (Pharmacia) as described (11). At the end of the reaction, unincorporated nucleotides were removed with a Sephadex G-50 spin column. Synthesized mRNA was recovered by ethanol precipitation and resuspended in water for oocyte injection.

Determination of Taurocholate Uptake into Oocytes. Oocytes were maintained in culture and uptake of [6-^{3}H]taurocholic acid (2.1–6.6 Ci/mmol; 1 Ci = 37 GBq; DuPont/New England Nuclear) was determined as described (8).

Sequence and Hydropathy Analysis of the Rat Liver Na^+/Bile Acid Cotransporting Polypeptide. Double-stranded cDNA of unidirectionally deleted clones (Erase-a-Base, Promega) was sequenced in both directions using the dideoxy-

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Abbreviation: prLNaBA, isolated cDNA clone encoding the rat liver Na^+/bile acid cotransport system.
†To whom reprint requests should be addressed.
§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M77479).

nucleotide chain-termination method (12). Ten oligonucleotides were synthesized as primers to fill in some gaps. Sequence analysis was performed with the DNA and protein sequence analysis program DNASIS/PROSIS (Pharmacia). Hydropathy analysis was performed by the method of Kyte and Doolittle (13) with a window of 19 amino acids. Putative membrane-spanning domains were determined according to Klein *et al.* (14).

Analysis of the Translation Product(s) in Oocytes Injected with prLNaBA-Derived mRNA. *X. laevis* oocytes were cultured for 3 days and then incubated for 16 hr at 19°C in Barth's solution containing 5% fetal calf serum and 100 μCi of L-[^{35}S]methionine per μl (1186 Ci/mmol; DuPont/New England Nuclear). Subsequently the oocytes were homogenized by repeated passages through a 25-gauge needle in 0.25 M sucrose supplemented with the protease inhibitors phenylmethylsulfonyl fluoride (1 mM) and antipain and leupeptin (1 μg/ml each). The total membrane material was pelleted at 100,000 × g_{av} for 15 min and resuspended in 0.1 M Na_2CO_3. After 30 min on ice, the samples were centrifuged as above and the final pellets were resuspended in homogenization buffer. For digestion with N-glycosidase F (Boehringer Mannheim), the oocyte membranes were boiled for 3 min in 95 μl of 0.25 M sodium phosphate containing 5 mM Na_2EDTA, 5 mM *o*-phenanthroleine, 0.1% (wt/vol) SDS, 1.0% (wt/vol) Nonidet P-40, and 10 mM 2-mercaptoethanol (pH 7.0). Two and one-half microliters (200 units/ml) of N-glycosidase F was added, and the samples were incubated for 18 hr at 37°C. The reaction was stopped by addition of 25 μl of 5× concentrated Laemmli sample buffer, and the samples were heated for 10 min at 50°C. Controls were incubated at 37°C without N-glycosidase F. Finally, the labeled proteins were separated by SDS/PAGE and visualized by autoradiography (24 hr) after enhancement with sodium salicylate (15).

***In Vitro* Translation of prLNaBA-Derived mRNA.** *In vitro* translation of cDNA-derived mRNA was performed with commercially available translation kits (Promega) containing either wheat germ extract, reticulocyte lysate, or reticulocyte lysate plus canine pancreatic microsomes. The conditions were as described by the manufacturer except for the additional presence of Triton X-100 (0.5% wt/vol) (16). Digestion with endoglycosidase H (endo-β-*N*-acetylglucosaminidase H) was performed as described (17). The labeled proteins were separated by SDS/PAGE and visualized by autoradiography (24 hr) after enhancement with sodium salicylate (15).

Northern Blotting. Samples of RNA from different tissues and species were separated by electrophoresis on a 1% agarose/formaldehyde gel, transferred to a nylon membrane (Hybond N, Amersham), and hybridized after UV crosslinking with an *Eco*RI fragment of the prLNaBA (nucleotides 261–1187) labeled by random priming. The blot was hybridized for 16 hr at 42°C in 50% formamide, 0.75 M NaCl/0.075 M sodium citrate at pH 7.0 (5× SSC), 5× Denhardt's solution, 0.5% SDS, and 200 μg of denatured salmon sperm DNA per ml. The filter was washed twice in 2× SSC containing 0.1% SDS at room temperature for 15 min and in 1× SSC containing 0.1% SDS at 59°C for 15 min. RNA size standards were stained with methylene blue.

RESULTS

From a cDNA library, a single positive clone (prLNaBA) was isolated, which, when transcribed and expressed in oocytes, conferred Na^+-dependent taurocholate uptake. Fig. 1 *Upper* shows that prLNaBA mRNA exhibited a markedly higher capacity to express Na^+-dependent taurocholate uptake into oocytes as compared to total rat liver poly(A)$^+$ RNA. Noninjected or water-injected (data not shown) oocytes did not demonstrate any Na^+ gradient-driven taurocholate uptake activity, indicating that native oocytes are devoid of this transport function.

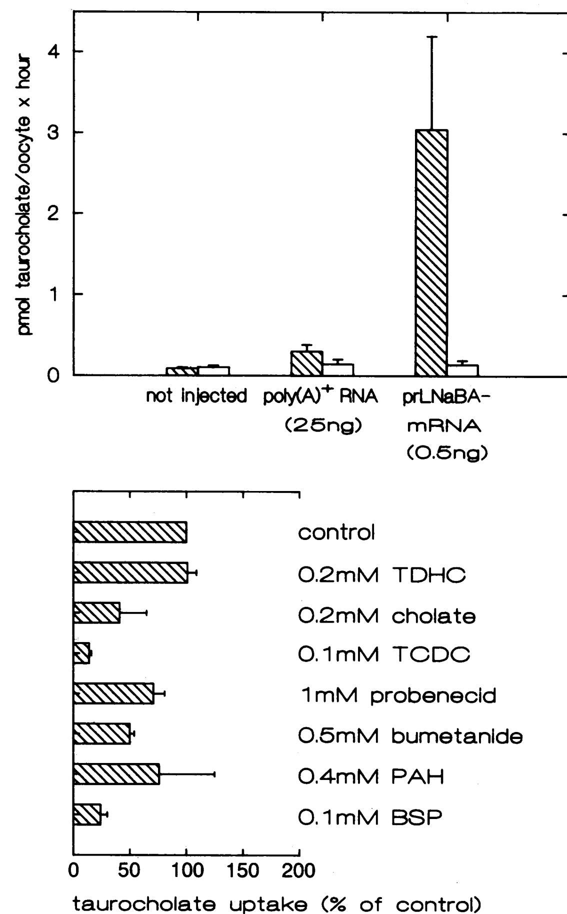


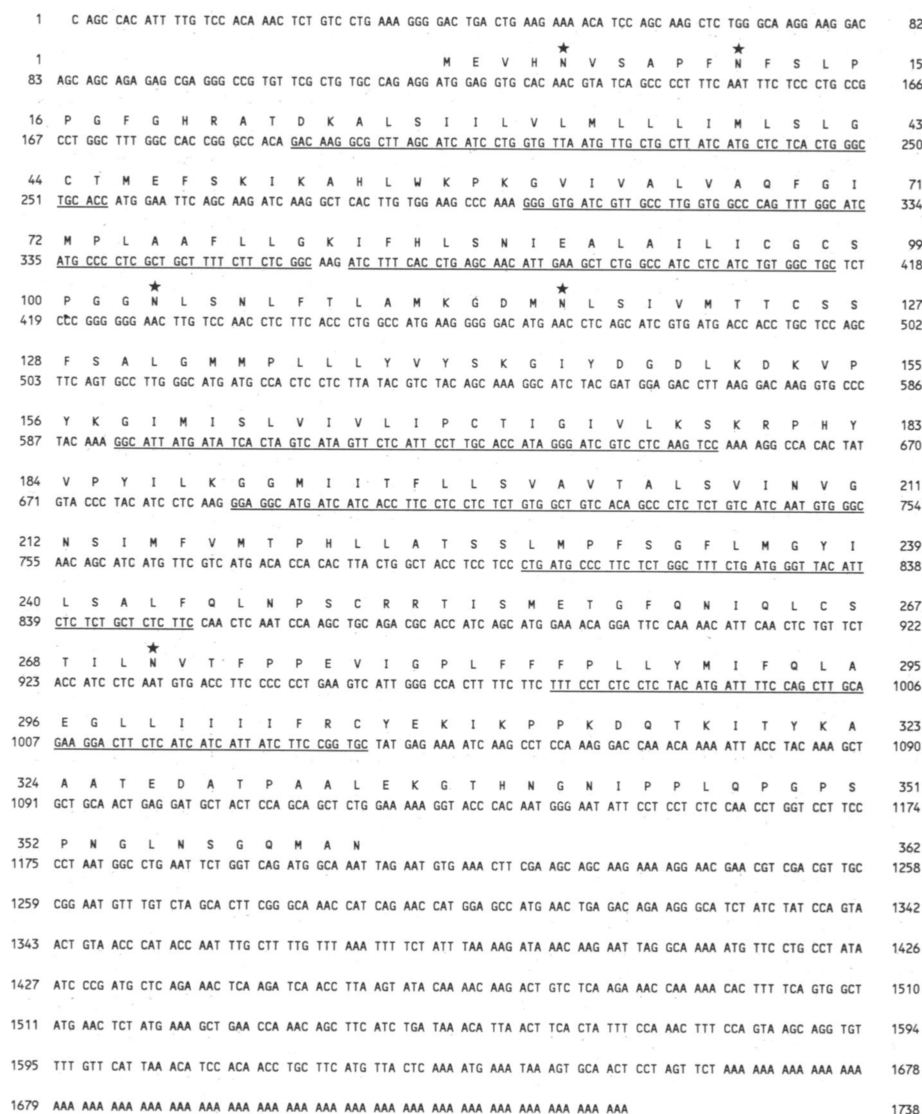
FIG. 1. Functional expression of prLNaBA-derived mRNA in oocytes. Oocytes were injected with 0.5 ng of prLNaBA-derived mRNA unless otherwise indicated. (*Upper*) Effect of Na^+ on [^{3}H]taurocholate uptake in oocytes. Oocytes either were not injected or were injected with the indicated amounts of total rat liver poly(A)$^+$ RNA or prLNaBA-derived mRNA, respectively. The cells were cultured for 5 days, and 1-hr taurocholate (17 μM) uptake values were determined in the presence of either 100 mM NaCl (hatched bars) or 100 mM choline chloride (open bars) as described (8). Values represent the means ± SD of 6–10 determinations in one of three oocyte preparations. (*Lower*) Effects of various organic anions on Na^+-dependent taurocholate uptake in prLNaBA mRNA-injected oocytes. Oocytes were cultured for 4 days after mRNA injection. Na^+-dependent taurocholate (17 μM) uptake (1-hr values) was determined in the absence (control = 100%) or presence of the indicated concentrations of the various compounds. Values represent the means ± SD of 20–30 determinations in three separate oocyte preparations. TDHC, taurodehydrocholate; TCDC, taurochenodeoxycholate; PAH, *p*-aminohippurate; BSP, bromosulfophthalein.

Interestingly, the cloned transport system was strictly dependent on the presence of extracellular Na^+ (Fig. 1 *Upper*). This finding proves that the previously demonstrated Na^+-independent saturable portion of taurocholate uptake into isolated hepatocytes and plasma membrane vesicles (1–4) has to be attributed to a separate, thus-far unidentified, hepatocellular transport system.

Various previous studies have repeatedly indicated that the hepatic Na^+/bile acid uptake system exhibits broad substrate specificity and transports various nonbile acid organic compounds as well (1–4, 8). Fig. 1 *Lower* indicates that the cloned Na^+/taurocholate uptake system was also inhibited by the physiological bile acids cholate and taurochenodeoxycholate, the anionic transport inhibitors bumetanide and

probenecid, and the amphipathic organic anion bromosulfophthalein. In contrast, the synthetic keto-bile acid taurodehydrocholate had no inhibitory effects, similar to previous findings in intact hepatocytes (18). Hence, the close similarity between the cis-inhibition pattern of the cloned transporter in oocytes and the native transporter in hepatic tissue strongly indicates that the identified cDNA indeed encodes the hepatic basolateral Na^+/bile acid cotransport system. This conclusion is further supported by recent kinetic experiments that indicated clear saturability of the expressed Na^+-dependent taurocholate uptake activity with an apparent K_m of ≈25 μM. This value is virtually identical to K_m values reported in intact hepatocytes (1) and in isolated basolateral rat liver plasma membrane vesicles (4).

The DNA sequence and the deduced amino acid sequence of the Na^+/taurocholate cotransporter are shown in Fig. 2 *Upper*. The total cDNA insert consists of 1738 nucleotides. There are two potential initiation sites at positions 122 and 257, both of which agree well with the Kozak consensus sequence (19). Based on the scanning model for translation (20) we assigned the initiation site to the first ATG codon at position 122. Consequently, the open reading frame extends over 1086 nucleotides, thus predicting a polypeptide of 362 amino acids with a molecular mass of ≈39 kDa. Within the 3′ untranslated region a potential poly(A) signal was identified 17 bases upstream of a stretch of A residues at position 1641, suggesting the isolation of a full-length clone. This conclusion is further supported by the identification of a 1.7-kb rat liver mRNA on a Northern blot (see Fig. 4). Computer-aided analysis of the cloned protein revealed five potential N-linked glycosylation sites and seven putative transmembrane-spanning domains (14). No cleavable signal sequence could



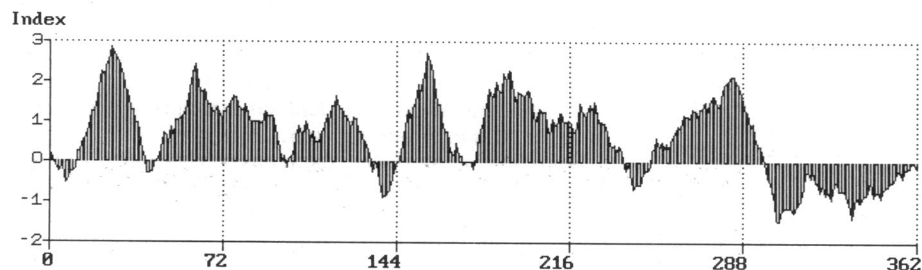


FIG. 2. Sequence and hydropathy analysis of the rat liver Na^+/bile acid cotransporter. (*Upper*) Nucleotide and deduced amino acid sequence of the prLNaBA cDNA. Putative membrane-spanning domains (14) are underlined. Potential N-linked glycosylation sites are marked by asterisks. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. (*Lower*) Hydropathy plot of the prLNaBA-encoded protein. Hydropathy plotting was performed by the method of Kyte and Doolittle (13) with a window of 19 amino acids. On the ordinate, hydrophobicity is indicated by positive numbers and hydrophilicity is indicated by negative numbers.

be identified (21). A search of the available data bases revealed a 30.9% identity of the cloned protein with human gene P3 protein (22) and up to 20% identities with the amino acid sequences of the Na^+-dependent glucose transporters (23–25) and the proline transporter of *E. coli* (26).

To directly identify the protein product encoded by the cloned cDNA, we next performed translation experiments *in vitro* and in oocytes. Fig. 3, lanes a–c, indicates that mRNA-injected oocytes specifically synthesized a 41-kDa protein, the molecular mass of which was decreased to ≈35 kDa after treatment of the membranes with N-glycosidase F. Thus, the 41-kDa protein appears to be a glycosylated form of the 35-kDa polypeptide. The same conclusion can be derived from the *in vitro* translation experiments (Fig. 3, lanes d–j). Hence, though in the absence of microsomes a 33-kDa protein was synthesized in the wheat germ extract and the reticulocyte lysate systems (Fig. 3, lanes f and g), addition of canine pancreatic microsomes resulted in a second protein band at 39 kDa (Fig. 3, lane h). This 39-kDa protein band shifted back to 33 kDa after digestion with endoglycosidase H (Fig. 3, lane i), indicating that it also represented a glycosylated form of the 33-kDa translation product. Furthermore, assuming a molecular mass contribution of ≈2000 per sugar chain on SDS gels, the results would be compatible with glycosylation of three of five glycosylation sites postulated above on the basis of the computer-aided sequence analysis (Fig. 2).

Aside from liver, Na^+-dependent taurocholate transport has also been demonstrated in brush border membranes of the ileum and kidney (27–30). Furthermore, the liver of all mammalian species (including man) exhibits highly active Na^+-dependent bile acid uptake. Therefore, we screened different rat tissues and livers from various species for possible homologies between these various bile acid transport systems. Fig. 4 indicates that, using low stringency conditions, an *Eco*RI fragment derived from the coding region of the cloned cDNA (nucleotides 261–1187) hybridized with a 1.7-kb mRNA of rat liver and kidney, whereas in rat duodenum and ileum a signal between 7.5 and 9.5 kb was detected. No hybridization signal was detected with mRNA from brain, skeletal muscle, lung, and heart (data not shown). Low stringency hybridization also resulted in the detection of liver mRNA of various species such as mouse, guinea pig, rabbit, and man (Fig. 4). Blot analysis under high stringency conditions showed a positive signal only with rat and mouse liver mRNA, indicating the highest homology of the transporters between these two species.

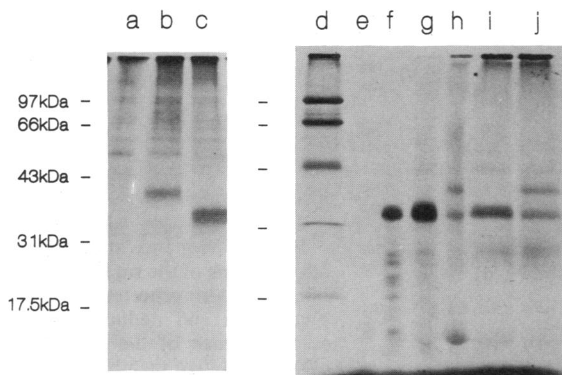


FIG. 3. Translation of prLNaBA-derived mRNA in oocytes and *in vitro*. Noninjected (controls; $n = 4$) and prLNaBA-derived mRNA injected ($n = 4$) *X. laevis* oocytes were cultured and incubated with L-[^{35}S]methionine, and the labeled oocyte membrane proteins were analyzed. Lane a, membranes of noninjected oocytes. Lane b, membranes of mRNA-injected oocytes. Lane c, membranes of mRNA-injected oocytes treated with N-glycosidase F. The *in vitro* translation results are illustrated in lanes d–j. Lane d, molecular mass standards. Lane e, wheat germ extract without mRNA. Lane f, wheat germ extract plus mRNA. Lane g, reticulocyte lysate plus mRNA. Lane h, reticulocyte lysate plus microsomes plus mRNA. Lane i, endoglycosidase H digestion (20 hr, 37°C) of the reticulocyte lysate/microsomal translation product. Lane j, control incubation (20 hr, 37°C) of the reticulocyte lysate/microsomal translation product in the absence of endoglycosidase H, confirming that the shift of the 39-kDa protein band to 33 kDa in lane i was indeed due to deglycosylation.

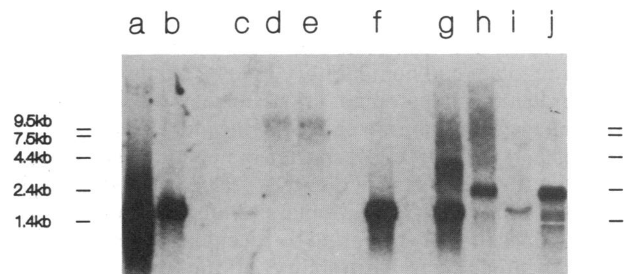


FIG. 4. Blot hybridization of RNAs from various rat tissues and from livers of different mammalian species. The following RNAs were analyzed under low stringency conditions: prLNaBA mRNA (0.1 ng; lane a), total rat liver RNA (2.5 μg; lane b), total rat kidney RNA (24 μg; lane c), rat duodenal mRNA (20 μg; lane d), rat ileal mRNA (20 μg; lane e), rat liver mRNA (0.5 μg; lane f), mouse liver mRNA (1 μg; lane g), rabbit liver mRNA (5 μg; lane h), human liver mRNA (30 μg; lane i), guinea pig liver mRNA (5 μg; lane j).

DISCUSSION

Using a functional expression cloning strategy (23, 24), we have isolated and characterized a cDNA encoding the basolateral Na^+/bile acid cotransporter of rat liver. The cloned transporter is strictly dependent on the extracellular presence of Na^+ and exhibits the same cis-inhibition pattern as the native transporter in hepatic tissue (Fig. 1). Though the cDNA sequence predicts a 39-kDa polypeptide with five potential N-linked glycosylation sites and seven putative transmembrane-spanning domains (Fig. 2), translation experiments *in vitro* and in oocytes indicated that the transporter is represented on SDS gels by a 33- to 35-kDa polypeptide, the molecular mass of which is increased by ≈6 kDa after its partial glycosylation *in vitro* (Fig. 3). Obviously, these apparent molecular masses of the translation products are lower than the 48- to 50-kDa values proposed as apparent molecular mass of the basolateral rat liver Na^+/bile acid symporter on the basis of hepatocyte photoaffinity labeling and protein isolation (5–7). However, lower apparent molecular masses of cloned and *in vitro* translated as compared to native transport proteins have also been observed for the Na^+-dependent and Na^+-independent glucose transporters (24, 31, 32). The reasons for these discrepancies in the apparent molecular masses of *in vitro* translated as compared to native membrane proteins are unknown but could be due to variable posttranslational modifications and/or different protein migration during SDS/PAGE. Hence, our data are not incompatible with the previously suggested molecular mass of the native transporter, and they do not necessary imply the involvement of a different, thus-far unidentified, Na^+/taurocholate cotransporting polypeptide. Alternatively, it might also be possible that hepatocytes localize more than one Na^+-dependent bile acid cotransport system at their basolateral membrane domain.

Searches of available data bases (National Biomedical Research Foundation Protein Sequence Database; Release 28.0) revealed a 30.9% identity in a 262-amino acid overlap between our cloned Na^+/bile acid cotransporter and the ubiquitous human gene P3 protein of unknown function(s)

(22). However, using the program BESTFIT (33) "only" 17–20% identities (51–53% similarities) were obtained with other Na^+-dependent transport systems such as the Na^+-dependent glucose cotransporter of rabbit and human intestine (23–25) and the proline transporter of *E. coli* (26). In addition, the sequence of the hepatic Na^+/bile acid cotransport system does not contain the Na^+-binding consensus sequence recently proposed as a possible common characteristic of all Na^+/solute symporters (25, 34).

Finally, the cloned cDNA hybridized with mRNA of rat tissues known to be active in bile acid transport (e.g., intestine, kidney) and with liver mRNA of various mammalian species (Fig. 4). Thus, the different sizes of the detected mRNA species do not necessarily imply different molecular masses of the encoded proteins, since they could also be explained by different lengths of 5′ and 3′ untranslated regions. Furthermore, since in rat kidney and rat ileum a 99-kDa polypeptide has been proposed to mediate apical Na^+-dependent taurocholate uptake (35–37), though no Na^+-dependent bile acid transport system was found in duodenal brush border membranes (27), the significance of the detected hybridization pattern might also be attributed to Na^+-independent bile acid transport systems (38). Hence, the availability of the cDNA encoding the hepatocellular Na^+/bile acid cotransport system might be of considerable help in future molecular characterization of bile acid transport systems of other tissues and species including small intestine and human liver.

We thank A. Werner and H. Murer for supplying us with rabbit liver mRNA. This study was supported by Swiss National Science Foundation Grants 32-9370.87 and 32-29878.90 and the Hartmann-Müller Foundation for Medical Research, University of Zurich/Switzerland.

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