Expression, transport properties, and chromosomal location of organic anion transporter subtype 3

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Walters, Holly C., Ann L. Craddock, Hisae Fusegawa, Mark C. Willingham, and Paul A. Dawson. Expression, transport properties, and chromosomal location of organic anion transporter subtype 3. Am J Physiol Gastrointest Liver Physiol 279: G1188–G1200, 2000.—The rat and mouse organic anion-transporting polypeptides (oatp) subtype 3 (oatp3) were cloned to further define components of the intestinal bile acid transport system. In transfected COS cells, oatp3 mediated Na+-independent, DIDS-inhibited taurocholate uptake (Michaelis-Menten constant \( K_m \) \( \cong 30 \mu M \)). The oatp3-mediated uptake rates and affinities were highest for glycine-conjugated dihydroxy bile acids. In stably transfected, polarized Madin-Darby canine kidney (MDCK) cells, oatp3 mediated only apical uptake of taurocholate. RT-PCR analysis revealed that rat oatp3, but not oatp1 or oatp2, was expressed in small intestine. By RNase protection assay, oatp3 mRNA was readily detected down the length of the small intestine as well as in brain, lung, and retina. An antibody directed to the carboxy terminus localized oatp3 to the apical brush-border membrane of rat jejunal enterocytes. The mouse oatp3 gene was localized to a region of mouse chromosome 12p12, where the human OATP-A gene was mapped, suggesting that rodent oatp3 is orthologous to the human OATP-A. These transport and expression properties suggest that rat oatp3 mediates the anion exchange-driven absorption of bile acids previously described for the proximal small intestine.

intestinal transport; brush-border membranes; organic anion transport; taurocholate

BILE ACIDS ARE SYNTHESIZED from cholesterol in the liver and secreted across the canalicular membrane. Along with other biliary constituents, bile acids enter the small intestine, where they facilitate the absorption of dietary lipids and fat-soluble vitamins. Bile acids are efficiently absorbed from the small intestine through a combination of passive absorption in the proximal small intestine and active absorption in the distal ileum. The absorbed bile acids are then returned to the liver in the portal circulation and resecreted into bile (20). The active absorption of bile acids in the terminal ileum is mediated by the well-characterized ileal apical Na+-bile acid transporter (ASBT) (31). This Na+- and potential-driven (47) transporter moves bile acids from the lumen of the small intestine across the apical brush-border membrane. The bile acids are then shuttled to the basolateral membrane and secreted by an anion exchange mechanism (46). Several observations suggest that the terminal ileum is the major site of bile acid reabsorption in humans and experimental animal models. These include the finding that there is little decrease in intraluminal bile acid concentration before the ileum (18) and the appearance of bile acid malabsorption after ileal resection (21). More recent studies using in situ perfused intestinal segments to measure bile acid absorption (32) have also demonstrated that ileal bile acid transport is a high-capacity system sufficient to account for the biliary output of bile acids. The consensus from these studies was that the ileal active transport system is the major route for conjugated bile acid uptake, whereas the passive or facilitative absorption down the length of the small intestine may be significant for unconjugated and some glycine-conjugated bile acids.

In contrast to the ileal absorption of bile acids, little is known regarding the mechanism for jejunal transport of bile acids. A fraction of the glycine conjugates and unconjugated bile acids are protonated and may be absorbed by passive diffusion across the apical brush-border membrane. However, there is emerging evidence for carrier-mediated transport of bile acids in the proximal small intestine in addition to membrane diffusion. In vivo uptake and cis-inhibition studies demonstrated bile acid specificity consistent with a facilitative carrier (3, 5). More recently, Amelsburg and co-workers (4) demonstrated an anion exchange mechanism for conjugated bile acid uptake in rat jejunal brush-border membranes. The properties of this transport system are similar to those ascribed to the family of organic-anion-transporting polypeptides (oatp).

The rat oatp family includes the bile acid and organic solute transporters oatp1 (22), oatp2 (35), oatp3 (1), and lst-1/oatp4 (8, 23), the renal methotrexate transporters OAT-K1 (38), and the PG transporter PGT (25).

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Two human members of the family are LST-1 (2), the human ortholog of lst-1, and OATP-A (28), for which a rodent ortholog has not been identified. The mouse ortholog of rat oatp1 was recently identified (16). Although several members of this family transport bile acids, Northern blot and RT-PCR analysis indicate that they are not expressed in the small intestine (22, 33, 38). To identify other bile acid transporters that may be involved in the apical uptake or basolateral secretion of bile acids, we employed low-stringency PCR and rapid amplification of cDNA ends (RACE) strategies to isolate a rat oatp3 cDNA from rat small intestine. The mRNA and protein expression properties, substrate specificity, and membrane localization determined in this work suggest that rat oatp3 is an intestinal apical transporter that participates in bile acid absorption down the length of the small intestine. The mouse oatp3 cDNA was also obtained by identification of mouse EST clones and subsequent 5’-RACE. Its chromosomal location suggests that oatp3 is the ortholog of human OATP-A.

MATERIALS AND METHODS

Materials. Male Sprague-Dawley rats were purchased from Zivic Miller Laboratories (Zelienopole, PA). [3H]taurocholic acid (2.0–2.6 Ci/mmol), [2,4,7,9-3H]folic acid (26.0 Ci/mmol), [3H(G)]digoxin (19 Ci/mmol) were purchased from NEN (University of California, San Diego) and were synthesized as described previously (12, 37). Inulin [14C]carboxylic acid (2–2259–2283, was used for PCR amplification with the rat ileal mRNA and protein expression properties, substrate specificity, and membrane localization determined in this work suggest that rat oatp3 is an intestinal apical transporter that participates in bile acid absorption down the length of the small intestine. The mouse oatp3 cDNA was also obtained by identification of mouse EST clones and subsequent 5’-RACE. Its chromosomal location suggests that oatp3 is the ortholog of human OATP-A.

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37°C. The cells were washed twice and then incubated for 30 min at 37°C in HBSS containing 10 μM [3H]taurocholate and the indicated concentrations of DIDS. The cells were then washed and harvested to determine cell-associated radioactivity, and protein, as described previously (10).

Transport assays in stably transfected MDCK cells. Stable expression of the human ileal apical Na\(^+\)-bile acid transporter and the rat oatp3 in MDCK cells was achieved as follows. On day 0, 100-mm plates were seeded with 5 × 10⁵ MDCK cells. On day 3, the cells were transfected with 5.5 μg of the transporter expression plasmid plus 0.5 μg of pSV₂Neo using the FuGENE 6 Transfection Reagent. On day 5, the cells were switched to medium B containing 700 μg/ml G-418. After selection for 15 days, individual colonies were picked, cells were switched to medium B containing 350 μg/ml G-418, and assayed for activity on day 2. The DIDS sensitivity of taurocholate transport was assayed as described for the transiently transfected COS cells, except the MDCK cells were incubated for 2.5 min at 37°C in HBSS containing 10 μM [3H]taurocholate. To determine the kinetics of bile acid transport, the cells were incubated with increasing concentrations of the radiolabeled bile acids in HBSS containing 137 mM choline for 5 min at 37°C. The cells were incubated in duplicate with six different concentrations of bile acid from 1 to 100 μM. The Michaelis-Menten constants (Kₘ) and maximal velocities (Vₘₐₓ) were determined by Eadie-Hofstee transformation. A similar procedure was used to examine the uptake of the non-bile acid substrates (folic acid, methotrexate, digoxin, and microcystin).

For membrane localization transport assays, untransfected MDCK, stably transfected MDCK-oatp3, or stably transfected MDCK-ASBT cells were seeded onto 12-mm Transwell filter inserts (Costar, Cambridge, MA) at 8.8 × 10⁴ cells/insert. The media in the apical and basolateral chambers were replaced every 2 days; on day 5, expression of the transfected cDNAs was induced by addition of media containing 10 mM sodium butyrate. Formation of a tight seal between the apical and basolateral chambers was monitored by transepithelial transport of inulin [¹⁴C]carboxylic acid (≈50 μM). After incubation for 30 min at 37°C, the diffusion of

### Table 1. Oligonucleotide primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Nucleotide Positions</th>
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</thead>
<tbody>
<tr>
<td><strong>Oatp1-Specific Primers</strong> (20) (GenBank Accession No. L19031)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RLOATP1</td>
<td>CAGGAGATGCTCTCTCTCG</td>
<td>1-22</td>
</tr>
<tr>
<td>RLOATP6</td>
<td>ATTCTGCTGGTGTTCTGG</td>
<td>531-510</td>
</tr>
<tr>
<td>OATP5</td>
<td>GCIAARTYIGARAAYTCN</td>
<td>645-664</td>
</tr>
<tr>
<td><strong>Oatp1-Degenerate Primer</strong></td>
<td></td>
<td>645-664</td>
</tr>
<tr>
<td>RBOATP1</td>
<td>GTCTCAGCATGAGAAGAGAC</td>
<td>21-40</td>
</tr>
<tr>
<td>RBOATP3</td>
<td>TCTACAGAACACATGGATCC</td>
<td>1450-1470</td>
</tr>
<tr>
<td>RBOATP4</td>
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<td>932-908</td>
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<td>RIOATP16</td>
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<td>Adapter primer</td>
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<td>UAP2</td>
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</tr>
<tr>
<td>NAP2</td>
<td>ACTATAGGCTCAGAGGGC-3’</td>
<td>645-664</td>
</tr>
<tr>
<td>UPM</td>
<td>CTATACGACTGATAGGATCGAC</td>
<td>99-77</td>
</tr>
<tr>
<td><strong>Cyclophilin-Specific Primers</strong> (14)</td>
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<tr>
<td>CYCF</td>
<td>CATGGTCAACCCACACGTT</td>
<td>1-20</td>
</tr>
<tr>
<td>CYCR</td>
<td>TGCGTCTGCTGCTGGCTG</td>
<td>99-77</td>
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<tr>
<td><strong>Mouse Oatp3-Specific Primers</strong> (GenBank Accession No. AF240694)</td>
<td></td>
<td></td>
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<td>Moatp3-3</td>
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<td>2242-2263</td>
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<td>ATGAGTGAGCACGAGGCTGCTGGAG</td>
<td>2257-2284</td>
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</table>
radiolabeled inulin across the cell monolayer from the apical and basolateral chambers was <1.5 and 0.3%, respectively. On day 6, the cells were assayed for \(^{3}H\)taurocholate uptake. The cell monolayers were washed with warm PBS, and each well was incubated with the indicated volumes of HBSS plus 10 \(\mu\)M \(^{3}H\)taurocholate added to the apical (0.5 ml) or basolateral (1.0 ml) chamber. After 30 min at 37°C, the medium was removed, and the cells were washed in ice-cold PBS and harvested to determine cell-associated radioactivity and protein (10).

\(oatp3\) mRNA tissue expression. RT-PCR analysis was used to compare the liver and small intestinal expression of \(oatp1\), \(oatp2\), and \(oatp3\) mRNA. Total cellular RNA was isolated using TRIzol Reagent (Life Technologies) as suggested by the manufacturer. Poly(A) RNA was prepared by oligo(dT)-cellulose chromatography using a MicroPoly(A) Pure Kit (Ambion, Austin, TX). The RNA was then reverse transcribed using a random-hexamer primer and a cDNA synthesis kit (SuperScript Kit, Life Technologies). For the PCR amplification of \(oatp2\) and \(oatp3\) (50 \(\mu\)l; 30 cycles at 94°C for 45 s, 65°C for 1 min, and 72°C for 2 min), the reactions contained 1 \(\mu\)l of cDNA, 0.7 \(\mu\)M primers, 0.2 mM dNTPs, 2 mM MgCl\(_2\), and 0.5 U of \(Taq\) polymerase. Oligonucleotide primers specific for \(oatp2\) (RBOATP1 and RBOATP4) and \(oatp3\) (ROATP1 and ROATP6) were used to PCR amplify the 1,726- and 972-bp products, respectively. The oligonucleotide primers ROATP1 and ROATP6 and an annealing temperature of 60°C were used to amplify a 531-bp fragment for \(oatp1\). For each primer pair, a reaction containing no cDNA template was included as a control for reagent contamination. Rat liver cDNA, small intestine cDNA, and mock cDNA reactions, to which no reverse transcriptase was added, were also PCR amplified using primers CYCPC and CYCIR for cyclophilin as a control for reagent contamination. Rat liver cDNA, small intestine cDNA, and genomic DNA were also PCR amplified as a control for genomic DNA contamination. The amplified products were separated on a 1% (wt/vol) agarose gel and visualized with ethidium bromide.

RNase protection assays (RPAs) were performed using an RPA II Kit (Ambion) and the indicated amount of RNA isolated from rat tissues. The antisense \(^{32}P\)-labeled riboprobes were prepared using a \(\alpha\)-\(\[^{32}\]P\)UTP (800 Ci/mmol) and T7 RNA polymerase (Maxiscript Kit, Ambion) from pBlueScript II KS-rat ASBT (nt 1–237) (47) that was linearized with EcoRI or from pBlueScript II KS-rat oatp3 (nt 1993–2404) that was linearized with PstI or NsiI. The sizes of the undigested probes/protected riboprobe fragments were 410/349 nt for \(oatp3\) (PstI-digested plasmid; \(oatp3\) nt 2051–2404), 165/116 nt for \(oatp3\) (NsiI-digested plasmid; \(oatp3\) nt 2288–2404), and 336/237 nt for ASBT (nt 1–237). A 295-nt oatp1 probe was also synthesized from pBlueScript II KS-rat oatp1 (nt 1–210) (22) that protected 86 nt of 5′-untranslated sequence and 124 nt of coding sequence, and a 487-nt oatp3 probe was synthesized that protected 237 nt of 5′-untranslated region and 149 nt of coding sequence. Unlabeled sense-strand transcripts were synthesized from the pBlueScript II KS-rat oatp3 and rat ASBT constructs using T3 RNA polymerase (Maxiscript Kit, Ambion) and used as standards for quantitation. The radiolabeled riboprobes were isolated by preparative electrophoresis on a 5% acrylamide-8 M urea denaturing gel and elution into 0.5 M ammonium acetate, 1 mM EDTA, and 0.2% SDS at 37°C. The individual antisense probes were hybridized with tissue RNA at 42°C for >12 h. Single-stranded riboprobe was digested with a mixture of RNase A and T1, and the protected products were resolved by electrophoresis on a 6% acrylamide-7 M urea denaturing gel. The dried gels were exposed to Amersham Hyperfilm with an intensifying screen at ~70°C. The amount of protected product was quantified using a PhosphorImager (Molecular Dynamics).

\(Antibody\) preparation. The cDNA coding for the carboxy-terminal 47 amino acids of rat oatp3 was PCR amplified, subcloned into pGEX 3X (Pharmacia), and sequenced. The glutathione \(S\)-transferase (GST)-oatp3 fusion protein was purified from \(Escherichia\) \(coli\) cytosol by glutathione affinity chromatography, as described elsewhere (42). Three New Zealand White rabbits were immunized with 500 \(\mu\)g of the GST-oatp3 fusion protein in Freund's complete adjuvant, and an Ig-enriched fraction was prepared from immune serum by precipitation with 50% ammonium sulfate. Affinity purification of the anti-oatp3 antibody was performed by sequential affinity chromatography using GST-coupled agarose to remove GST-specific antibodies followed by GST-oatp3-coupled agarose according to the manufacturer’s instructions (AminoLink Immobilization Kit, Pierce, Rockford, IL). The affinity-purified antibody was stored at ~70°C and subjected to only one freeze-thaw cycle.

\(Immunolocalization\). Sections (6 \(\mu\)m) of rat intestine or 30-mm dishes of COS cells were fixed in 3.7% formaldehyde, washed in PBS, and incubated in blocking solution (PBS containing 1% BSA and 0.1% saponin) for 10 min at room temperature. The sections were then incubated with 2.9 \(\mu\)g/ml affinity-purified anti-oatp3-GST fusion protein antibody or preimmune Ig (50% ammonium sulfate-precipitated fraction) in the blocking solution for 0.5–1.5 h at room temperature. The sections were washed four times in PBS and once in the blocking solution. The sections were then incubated with 25 \(\mu\)g/ml rhodamine-conjugated goat anti-rabbit IgG secondary antibody (Jackson Immunoresearch Laboratories) in blocking solution for 0.5–1.5 h at room temperature. After four washes with PBS, the sections were fixed in 3.7% formaldehyde for 10 min and viewed using a Zeiss Axioscope 2 fluorescence microscope equipped with rhodamine epifluorescence optics. Images were captured using a Dage 300 charge-coupled device digital camera. For each experiment, a section or dish was incubated with the secondary antibody alone to determine background fluorescence.

Identification of the mouse \(oatp3\) cDNA. Oligonucleotide primers Moatp3-6 and Moatp3-4 were designed on the basis of the 3′-untranslated sequence of two mouse EST clones (GenBank accession nos. AA690580 and AA612385) and used to obtain the 5′-untranslated sequence and complete coding region sequence of the mouse oatp3 cDNA by 5′-RACE (Smart Race Kit, Clontech). Briefly, first-strand cDNA was synthesized from 1 \(\mu\)g of mouse brain poly(A) RNA (Clontech) using an oligo(dT) primer. During the first-strand synthesis, a 5′-adaptor sequence was added to the cDNA. The primer pairs UP/Moatp3-6 and NUP/Moatp3-4 were employed in the primary and secondary PCR amplifications, respectively, of the cDNA. The PCR-amplified product was isolated from a 0.8% (wt/vol) agarose gel, subcloned into pBluescript II KS, and sequenced using a Perkin-Elmer ABI Prism 377 sequencer.

Chromosomal localization of the mouse \(oatp3\) gene. The \(oatp3\)-specific oligonucleotide primers Moatp3-3 and Moatp5-5 were designed on the basis of the 5′-untranslated sequence of the mouse \(oatp3\) and used to screen a whole mouse genome/hamster radiation hybrid panel (Research Genetics) by PCR amplification. The reactions contained 18.65 ng of the panel DNA, 50 mM KCl, 10 mM Tris-HCl, pH 8.2, 1.5 mM MgCl\(_2\), 0.2 mM dNTPs, each oligo at 0.4 \(\mu\)M, and 0.5 U of \(Taq\) polymerase. Reactions containing mouse or hamster genomic DNA or no template were also included as controls. The reactions underwent 35 cycles in a GeneAmp PCR System 9700 (PE Applied Biosystems, Foster City, CA).
at 94°C for 40 s, 59°C for 40 s, and 72°C for 40 s. The PCR products were analyzed on 1% TAE agarose gels. The hybrid panel DNA samples were scored as positive or negative for the 216-bp mouse oatp3 product, and the retention pattern was submitted to the Jackson Laboratories Mouse Radiation Hybrid Database for analysis.

RESULTS

Isolation of oatp3 cDNA from rat intestine. To isolate additional components of the intestinal bile acid transport system, a 297-bp fragment of a novel oatp was identified from rat ileal cDNA using oatp2-specific oligonucleotide primers and low-stringency PCR. A combination of RACE and PCR amplification was then used to obtain the complete 2,765-bp cDNA, designated oatp3 (GenBank accession no. AF083469). This complete intestinal oatp cDNA includes a 237-bp 5’-untranslated region, a 2,010-bp open reading frame, and a 518-bp 3’-untranslated region. The 3’-untranslated sequence contains a canonical polyadenylation signal (AATAAA) located at position 2747 and is followed by a 17-nt poly(A) tail. The initiator methionine lies in an appropriate consensus for initiation of translation (26) and is preceded by an in-frame stop codon. The three upstream ATG codons located in the predicted 5’-untranslated region are closely followed by in-frame stop codons. The 2,010-bp open reading frame encodes a 670-amino acid polypeptide with a calculated molecular mass of 74,496 Da. The intestinal oatp cDNA sequence was nearly identical to the oatp3 that was identified from rat retina (1) (GenBank accession no. AF041105), except for five nucleotide changes at positions 334, 401, 445, 1864, and 1865. These nucleotide substitutions result in four amino acid differences between the retinal and ileal oatp3 clones (retinal clone residue listed first: Q33K, I55T, F70L, and K543E). These differences may be due to PCR errors, sequencing errors, or polymorphisms; in each case, the nucleotide sequence reported here changed the predicted amino acid to a residue that matched the oatp1/2 consensus. A search of available nucleic acid databases revealed that the rat oatp3 protein sequence is 81%, 82%, 77%, 40%, and 36% identical to that of rat oatp1 (22) (GenBank accession no. L19031), oatp2 (35) (GenBank accession no. U88036), OAT-K1 (38), (GenBank accession no. D79981), lst-1 (23) (GenBank accession no. AF147740), and PGT (25) (GenBank accession no. M64862), respectively. Hidden Markov model analysis (http://www.enzim.hu/hmmtop) of the protein sequence predicted a topology with 12 transmembrane domains, similar to other members of the oatp family (45).

Transport properties of oatp3. To examine the kinetics of oatp3-mediated bile acid uptake, transfected COS cells were incubated with increasing concentrations of [3H]taurocholate for 2.5 min at 37°C. Previous studies had shown that transport of [3H]taurocholate by oatp3 was linear up to 5 min (Fig. 1, inset). The transport of [3H]taurocholate by oatp3-transfected COS cells was saturable (Fig. 1), with an apparent Km of 30 μM and a Vmax of 240 pmol·min⁻¹·mg protein⁻¹. To compare the bile acid substrate specificity of the rat oatp3 and ASBT, transfected COS cells were incubated for 2.5 min with 10 μM radiolabeled bile acid, and uptake was quantitated. Rat oatp3 and ASBT transport all the major species of bile acids, but ASBT transported them at a greater rate (Fig. 2). Oatp3 transported glycine-conjugated bile acids more rapidly than taurine conjugates. This preference is particularly evident for the glycine conjugates of deoxycholate and ursodeoxycholate, which exhibited initial uptake rates that were 3.6- and 2.6-fold, respectively, greater than the corresponding taurine conjugates. Also, oatp3 transported the dihydroxy bile acids more rapidly than the trihydroxy bile acids for glycine and taurine conjugates (3.5-, 2-, and 3.9-fold over glycodeoxycholate, glycocholate, and glycodihydroxy bile acids, respectively). In contrast, ASBT showed neither of these trends for bile acid transport rate.

The kinetics of oatp3-mediated uptake of the different bile acids were determined using stably transfected MDCK cells (Table 2). The Km values for the conjugated bile acids were more influenced by the number of hydroxyl groups on the steroid nucleus than by the type of conjugation. The dihydroxy bile acids had Km values ranging from 4.3 to 7.0 μM, and taurocholate and glycocholate had Km values of 20.9 and 15.4 μM, respectively. The oatp3-mediated uptake of four non-
bile acid substrates transported by other members of the oatp family (14, 35, 38) was also examined using the MDCK-oatp3 stable cells. In these studies, oatp3 exhibited narrower substrate specificity and did not show appreciable transport of the nutrient folate, the cyanobacterial toxin microcystin, or the drugs methotrexate and digoxin (data not shown).

Transport mediated by previously identified members of the oatp family is Na\(^+\) independent (22, 29, 35, 38) and sensitive to the general anion transport inhibitor DIDS (29, 33, 38). To determine whether oatp3 shares these properties, oatp3-transfected COS cells were incubated in a modified HBSS containing 137 mM Na\(^+\) or an equal concentration of K\(^+\). Oatp3-mediated \(^{[3H]}\)taurocholate uptake was similar in the presence or absence of Na\(^+\) (Fig. 3, inset). Uptake of \(^{[3H]}\)taurocholate by oatp3 stably transfected MDCK cells (Fig. 3) or by oatp3 transiently transfected COS cells (data not shown) was inhibited by preincubation with DIDS; half-maximal inhibition was observed at \(\sim 115 \mu M\).

Analysis of oatp3 membrane expression. The membrane localization of oatp3 was examined in the stably transfected MDCK cells. Figure 4 shows the cellular accumulation of \(^{[3H]}\)taurocholate from the apical and basolateral chambers in monolayers of the parental MDCK cells and MDCK cells stably transfected with the human ASBT (MDCK-ASBT) and the rat oatp3 (MDCK-oatp3). The parental MDCK and MDCK-ASBT cells were examined as a negative and positive control, respectively. Taurocholate uptake was expressed only on the apical surface for the human ASBT-transfected MDCK cells (Fig. 4A), in agreement with previous studies of the rat ASBT (43). These findings indicate that the transfected MDCK cells reproduced the normal trafficking for this bile acid transporter.

Table 2. Kinetics of bile acid uptake by MDCK-oatp3 cells

<table>
<thead>
<tr>
<th>(^{[3H]})Bile Acid</th>
<th>Apparent (K_m), (\mu M)</th>
<th>(V_{max}), pmol-min(^{-1})-mg protein(^{-1})</th>
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<tr>
<td>Cholate</td>
<td>8.8</td>
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<td>110.6</td>
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<td>Glycocholate</td>
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</tr>
<tr>
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<td>106.7</td>
</tr>
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</tr>
<tr>
<td>Tauroursodeoxycholate</td>
<td>6.6</td>
<td>43.6</td>
</tr>
<tr>
<td>Glycusodeoxycholate</td>
<td>5.3</td>
<td>44.2</td>
</tr>
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</table>

\(K_m\), Michaelis constant; \(V_{max}\), maximal velocity. Madin-Darby canine kidney (MDCK) cells were stably transfected with the rat oatp3 and assayed for uptake of radiolabeled bile acid as described in the legend to Fig. 1.

Analysis of oatp3 membrane expression. The membrane localization of oatp3 was examined in the stably transfected MDCK cells. Figure 4 shows the cellular accumulation of \(^{[3H]}\)taurocholate from the apical and basolateral chambers in monolayers of the parental MDCK cells and MDCK cells stably transfected with the human ASBT (MDCK-ASBT) and the rat oatp3 (MDCK-oatp3). The parental MDCK and MDCK-ASBT cells were examined as a negative and positive control, respectively. Taurocholate uptake was expressed only on the apical surface for the human ASBT-transfected MDCK cells (Fig. 4A), in agreement with previous studies of the rat ASBT (43). These findings indicate that the transfected MDCK cells reproduced the normal trafficking for this bile acid transporter. Cellular taurocholate accumulation from the apical chamber was 26-fold greater for the MDCK-oatp3 cells than for...
the parental MDCK cells (Fig. 4B). In contrast, taurocholate uptake from the basolateral chamber was similar in the parental MDCK and oatp3 stably transfected MDCK cells.

Analysis of rat oatp3 tissue expression. RT-PCR and RPA were used to investigate the expression of oatp3 in liver and intestine. As shown in Fig. 5A, oatp1 and oatp2 mRNAs were detected in rat liver, but not rat small intestine, cDNA. In contrast, oatp3 was readily detected in small intestine, with only a faint but reproducible signal amplified from rat liver cDNA. RPA analysis of the horizontal gradient of oatp3 mRNA expression down the length of the small intestine is shown in Fig. 5B. The assay employed a 32P-labeled oatp3 probe containing 199 nt of coding sequence and 150 nt of 3'-untranslated region. Oatp3 mRNA was present at ~0.04 pg/µg total RNA and was expressed at similar levels down the length of the small intestine. This oatp3 probe could potentially cross-hybridize with other members of the family. However, no smaller cross-hybridizing products were detected with the oatp3 probe, nor was a protected fragment detected with an oatp1-specific probe in the RPA analysis of the intestinal RNAs (data not shown). The absence of cross-hybridizing related oatp transcripts in small intestine agrees with the RT-PCR analysis (Fig. 5A) and previous studies of OAT-K1 mRNA expression (38).

In contrast to oatp3 mRNA, rat ASBT mRNA was expressed only in the terminal ileum, as reported previously (9). In addition to the protected ASBT fragment of the expected size (237 nt, including 106 nt of 5’-untranslated region and 131 nt of coding sequence), a smaller 210-nt fragment was also detected that may result from alternative transcription start sites (Dr. Ben Shneider, personal communication). The amount of ASBT mRNA expressed in rat terminal ileum (segment 9) was ~162 pg/µg total RNA.

RPA analysis of oatp3 mRNA expression in other rat tissues is shown in Fig. 6. For this analysis, a 32P-labeled probe encompassing 116 nt of only divergent 3’-untranslated region was used. This region was selected since preliminary results with coding region-containing probes indicated significant cross-hybridization with related oatp mRNA in some tissues. For example, no protected product was observed using the 3’-untranslated region probe for RPA analysis of kidney RNA (Fig. 6). However, an RPA probe containing oatp3 coding and 5’-untranslated region sequence protected a product that was significantly smaller than the oatp3 predicted size. The size of the protected fragment matched a segment of the coding region portion of the probe that shared extensive sequence identity (>90%) with related oatp cDNA clones (data not shown). The oatp3 mRNA was readily measured in rat brain and lung (~0.4 pg/µg total RNA). The oatp3 mRNA was also detected at levels just above background in spleen, liver, and hepatocyte but was undetectable by RPA in heart and kidney. Thus 5’- and 3’-probes were unable to detect oatp3 mRNA in kidney. As shown previously (1), the oatp3 mRNA was also expressed in retina (~0.04 pg/µg total RNA). However, in contrast to the previous report, retinal oatp3 mRNA was present at levels significantly below that of whole brain. In addition, since human ASBT EST clones (GenBank accession nos. AA504910 and AA504859) have been reported from a human fetal retinal cDNA library, ASBT expression was also investigated in rat retina. Unlike oatp3, ASBT was not detectable in retinal RNA by RPA (data not shown), suggesting that the human ASBT sequences were obtained from contaminating genomic clones.

The affinity-purified antibody, designated αGST-oatp3, was used for immunolocalization of the oatp3 protein in transfected cells and in intestinal tissue. Figure 7 shows that αGST-oatp3 strongly reacted with the oatp3-transfected cells (Fig. 7F). The antibody also cross-reacted with oatp1 (Fig. 7B) but not oatp2 (Fig. 7D). The lack of cross-reactivity with oatp2 was not due to low expression in the transfected COS cells. Parallel dishes of oatp1-, oatp2-, and oatp3-transfected COS cells exhibited similar levels of [3H]taurocholate uptake (data not shown). The oatp3 immunolocalization studies then focused on rat small intestine, where oatp1 is not expressed (Fig. 5A). Cryosections from rat jejunum were incubated with an ammonium sulfate-precipitated fraction from preimmune serum or the affinity-purified αGST-oatp3 (Fig. 8). The preimmune antibody had no reactivity (Fig. 8A), whereas αGST-
addition, the contig and the rat oatp3 share ~81% identity along the length of their 3'-untranslated regions, whereas the 3'-untranslated regions of the other oatp family members shares <50% identity. At the amino acid level, the contig shares 92% identity with the rat oatp3 and <78% identity with other members of the oatp family. This very high degree of sequence identity indicates that the EST clones represent the mouse ortholog of the rat oatp3. The sequence of the complete mouse oatp3 cDNA (GenBank accession no. AF240694) was obtained by 5'-RACE. The cDNA includes 94 bp of 5'-untranslated sequence, a 2,010-bp coding region, and 434 bp of 3'-untranslated region. The 3'-untranslated sequence included a canonical polyadenylation signal (AATAAA) located at position 2519 and was followed by a 22-nt poly(A) tail. The initiator methionine lies in an appropriate consensus for initiation of translation (26) and is preceded by an in-frame stop codon. An upstream ATG codon is closely followed by an in-frame stop codon. The 2,010-bp open reading frame encodes a 670-amino acid polypeptide with a calculated molecular mass of 74,758 Da and a putative topology of 12 transmembrane domains (45). The predicted mouse oatp3 protein sequence is 90, 80, 82, 78, 43, and 35% identical to the rat oatp3, oatp1 (22), oatp2 (35), oatk1 (38), ist-1 (23), and PGT (25), respectively (Table 3).

Chromosomal localization of the mouse oatp3 gene. To determine the chromosomal location of the mouse oatp3 gene, two mouse oatp3 3'-untranslated region-specific oligonucleotide primers were used to screen a

![Fig. 5. Intestinal expression of rat oatp3. A: poly(A) RNA (2 µg) from rat liver (L; lanes 2, 5, and 8) and small intestine mucosal cells (I; lanes 3, 6, and 9) was reverse transcribed and used for PCR amplification with oligonucleotide primers specific for rat oatp1, oatp2, or oatp3. A separate reaction was performed in the absence of cDNA (H2O; lanes 1, 4, and 7) as a control for contamination. Amplified products were separated on a 1% (wt/vol) agarose gel and visualized with ethidium bromide. A fragment of cyclophilin was also amplified using a PhosphorImager; the absolute amount of specific transcript was estimated from a standard curve using in vitro transcribed sense-strand standards analyzed in parallel. B: expression of oatp3 and ASBT mRNA along the longitudinal axis of the small intestine. The entire rat small intestine was divided into 9 equal segments and used to prepare mucosal scrapings for RNA isolation. Each RNA sample was incubated in the absence (lanes 1) or presence (lanes 2–11) of a mixture of RNase A and T1, and the protected products were resolved by electrophoresis on a 6% acrylamide-7 M urea denaturing gel. The amount of protected riboprobe was quantitated using a PhosphorImager; the absolute amount of specific transcript was estimated from a standard curve using in vitro transcribed sense-strand standards analyzed in parallel.

![Fig. 6. Tissue distribution of rat oatp3 mRNA. Top: yeast tRNA (10 µg; lanes 1, 2, 10, and 11) and total RNA (50 µg; lanes 3–9; 10 µg; lanes 12 and 13) from the indicated rat tissues were hybridized with a rat oatp3 32P-labeled riboprobe corresponding to only 3'-untranslated region sequence (nt 2288–2404). The hybridization reaction was incubated in the absence (lanes 1 and 10) or presence (lanes 2–9 and 11–13) of a mixture of RNase A and T1. The protected products were resolved by electrophoresis on a 6% acrylamide-7 M urea denaturing gel. The amount protected riboprobe was quantitated using a PhosphorImager; the absolute amount of specific transcript was estimated from a standard curve using in vitro transcribed sense-strand standards analyzed in parallel. Bottom: parallel samples of RNA (5 µg; lanes 3–9; 2 µg; lanes 12 and 13) were hybridized with a 32P-labeled actin riboprobe and analyzed as described for top panel.

![Fig. 7. Chromosomal localization of the mouse oatp3 gene. A: the oatp3 gene, two mouse oatp3 3'-untranslated region-specific oligonucleotide primers were used to screen a

oatp3 stained the apical membrane of epithelial cells (Fig. 8, B and C). Goblet cells (arrows in Fig. 8, B and C) were not labeled.

Isolation of oatp3 cDNA from mouse brain. A search of the EST database yielded two overlapping mouse EST clones, one from skin and one from mammary gland (GenBank accession nos. AA690580 and AA612385), with significant identity to rat oatp3. A contig of the two clones encompassed 499 bp of coding region and 434 bp of 3'-untranslated sequence. At the nucleotide level, the coding region of this contig shares 92% identity with the rat oatp3 coding region and <86% identity with other members of the family. In

![Fig. 7. Chromosomal localization of the mouse oatp3 gene. A: the oatp3 gene, two mouse oatp3 3'-untranslated region-specific oligonucleotide primers were used to screen a
mouse/hamster radiation hybrid panel by PCR. The retention patterns were submitted to the Jackson Laboratory Mouse Radiation Hybrid Database, where linkage analysis was performed. An optimal logarithm of odds score of 13.0 was obtained with marker D6Mit58. This localizes the mouse oatp3 gene (locus symbol Slc21a7) to a region of mouse chromosome 6 syntenic with human chromosome 12p12. The human OATP-A gene has been localized to this region of chromosome 12 by radiation hybrid analysis (28) and fluorescent in situ hybridization (30), suggesting that OATP-A is the human ortholog of oatp3. Interestingly, the human LST-1 gene (aliases: OATP-C, OATP2, and SLC21A6) is encoded by a BAC clone (GenBank accession no. AC022335) also mapping to human chromosome 12p (www.hgsc.bcm.tmc.edu/seq_data/project-table.cgi?submit=Run&maplocation=Human+12p). This observation raises the possibility that a cluster of oatp-like genes is encoded in this chromosomal region and that another human ortholog may exist for oatp3.

A comparison of the amino acid sequences for OATP-A and rat and mouse oatp3 (Fig. 9) reveals identities of 72% shared with both rodent polypeptides. This high degree of identity suggests that oatp3 and OATP-A are orthologous.

**DISCUSSION**

Bile acid absorption in the jejunum was originally considered to occur only by passive diffusion across the brush-border membrane (11). However, recent studies in a variety of species indicate that facilitated transport is present in jejunum (3–5). This study reports the cloning and analysis of a strong candidate for the facilitative intestinal bile acid transporter oatp3.

The rat oatp3 cDNA shares considerable identity with other members of the rat oatp family, including mouse/hamster radiation hybrid panel by PCR. The retention patterns were submitted to the Jackson Laboratories Mouse Radiation Hybrid Database, where linkage analysis was performed. An optimal logarithm of odds score of 13.0 was obtained with marker D6Mit58. This localizes the mouse oatp3 gene (locus symbol Slc21a7) to a region of mouse chromosome 6 syntenic with human chromosome 12p12. The human OATP-A gene has been localized to this region of chromosome 12 by radiation hybrid analysis (28) and fluorescent in situ hybridization (30), suggesting that OATP-A is the human ortholog of oatp3. Interestingly, the human LST-1 gene (aliases: OATP-C, OATP2, and SLC21A6) is encoded by a BAC clone (GenBank accession no. AC022335) also mapping to human chromosome 12p (www.hgsc.bcm.tmc.edu/seq_data/project-table.cgi?submit=Run&maplocation=Human+12p). This observation raises the possibility that a cluster of oatp-like genes is encoded in this chromosomal region and that another human ortholog may exist for oatp3.

A comparison of the amino acid sequences for OATP-A and rat and mouse oatp3 (Fig. 9) reveals identities of 72% shared with both rodent polypeptides. This high degree of identity suggests that oatp3 and OATP-A are orthologous.
oatp1, oatp2, and OAT-K1. The sequence identity extends throughout the coding region and ends abruptly 23–53 nt upstream of the initiator methionine and 41–50 nt downstream of the stop codon. The divergent 3′- and 5′-untranslated regions indicate that oatp3 is a distinct gene product and not an alternatively spliced isoform of a previously identified oatp. The 74-kDa protein encoded by this oatp3 cDNA also shares considerable structural identity with the other known rat oatps. It is predicted to be a polytopic membrane protein with a cytoplasmic amino terminus, 12 putative transmembrane segments, and a cytoplasmic carboxy terminus. The predicted protein contains four potential glycosylation sites, three of which are conserved among the rat oatps.

When expressed in transfected COS cells, rat oatp3 mediates Na⁺-independent, saturable taurocholate uptake with an apparent \( K_m \) of 30 \( \mu M \) and a \( V_{max} \) of 240 pmol·min⁻¹·mg protein⁻¹. This \( K_m \) is similar to that reported for the retinal library-derived oatp3 clone expressed in Xenopus oocytes (18 \( \mu M \)) (1), for oatp1 expressed in Xenopus oocytes (50 \( \mu M \)) (29), HeLa cells (27 \( \mu M \)) (24), and Chinese hamster ovary (CHO) cells (32 \( \mu M \)) (13), and for oatp2 (34 \( \mu M \)) (35) and human OATP (60 \( \mu M \)) (28) expressed in Xenopus oocytes. In addition, the apparent \( K_m \) is very similar to the value of 54 \( \mu M \) determined for the taurocholate transport by rat jejunal brush-border membrane vesicles (4).

In the rat jejunal brush-border membrane vesicle study, the dihydroxy bile acid taurochenodeoxycholate was transported at twice the rate of the trihydroxy bile acid taurocholate. Also, the \( K_m \) of taurocholate uptake by the vesicles was approximately twofold greater than the \( K_m \) of taurochenodeoxycholate uptake (4). Previous studies of jejunal absorption have also shown a preference for glycine conjugates and dihydroxy bile acids in humans (19, 40), rabbits (3), and guinea pigs (5). These substrate specificities and relative affinities are similar to those exhibited by rat oatp3 in transfected cells. The greater influence of steroid nucleus hydroxylation than the conjugation on relative affinities as measured in the MDCK-oatp3 stable cells is similar to observations of oatp1-mediated uptake in stably transfected CHO cells (13). The rat ASBT did not exhibit the trends in substrate specificity observed for rat oatp3. ASBT transported taurochenodeoxycholate and taurocholate at the same rate, in contrast to the observations of the rat jejunal brush-border membrane vesicles. These results support the hypothesis that rat jejunal bile acid uptake is mediated by a distinct transporter and is not due to low levels of ASBT expression in the proximal small intestine (4, 5).

The demonstration of Na⁺-independent but bicarbonate trans-stimulated taurocholate uptake by rat jejunal brush-border membrane vesicles (4) also supports the presence of a bile acid transporter distinct from the ASBT. Like other oatps (22, 25, 28, 35, 38), rat oatp3-mediated transport is Na⁺ independent. The oatp3 may also be trans-stimulated by bicarbonate. Oatp3 shares extensive similarity with oatp1, which has been shown to function as a taurocholate/bicarbonate exchanger (39). Taurocholate uptake by the rat jejunal brush-border membrane vesicles (4) and by oatp3 is sensitive to the general anion transport inhibitor DIDS. Preincubation with 250 \( \mu M \) DIDS inhibited rat brush-border membrane taurocholate uptake by 81% (4). In rat oatp3-transfected COS and MDCK cells, the same concentration of DIDS inhibited taurocholate uptake to a comparable degree (59%).

The similar bile acid transport kinetics, substrate specificity, Na⁺ independence, and DIDS sensitivity shared by jejunal brush-border membrane vesicles and oatp3 suggest that oatp3 is the carrier responsible for the jejunal absorption of bile acids described by Amelberg and colleagues (4, 5). Furthermore, the apical localization of oatp3 in MDCK cells, a model system for epithelial cell polarity, suggested that the transporter is expressed on the appropriate membrane to act as the brush-border membrane Na⁺-independent bile acid transporter.

The expression of oatp3 mRNA down the length of the small intestine is consistent with the hypothesis that oatp3 is the facilitative carrier responsible for intestinal passive absorption of bile acids. The other known bile acid-transporting rat oatps, oatp1 and oatp2, are not candidates, because they are not detected in the small intestine by Northern blot analysis (22), RT-PCR (38), or RPA. Staining of the apical membranes of jejunal epithelial cells by the oatp3 antibody confirmed that the protein is expressed in the small intestine on the jejunal apical brush-border membrane. The weak but reproducible staining by the anti-oatp3 antibody as well as the low level of oatp3 mRNA expression in the small intestine compared with ASBT may explain the previous difficulty in distinguishing Na⁺-independent facilitated transport from membrane

### Table 3. Percent amino acid identities shared by members of the oatp family

<table>
<thead>
<tr>
<th></th>
<th>Human OATP-A</th>
<th>Rat oatp1</th>
<th>Mouse oatp1</th>
<th>Rat oatp2</th>
<th>Rat oatp3</th>
<th>Mouse oatp3</th>
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</table>

Amino acid sequences were compared using the Blastn matrix OBLOSUM 62 program without a filter (17).
diffusion of bile acids (3, 5, 11). Further studies to quantitate the oatp3 protein and activity in the small intestine are required to estimate the potential capacity of this system in humans and experimental animal models. The expression of oatp3 mRNA in rat brain, lung, and retina in addition to small intestine indicates that oatp3 must transport other solutes in addition to bile acids. This is not surprising given the broad substrate specificity demonstrated for the related oatps (13, 34). Oatp3 expressed in *Xenopus* oocytes has been shown to transport the triiodothyronine and thyroxine forms of thyroid hormone (1). Although other substrates for oatp3 remain to be determined, its intes-
coding sequence that is highly conserved between the oatp family members. In contrast, the RNase protection probe used in this study was derived solely from divergent 3'-untranslated region sequence. Unfortunately, it has not been possible to directly compare the sequences of the probes used in these studies, since the untranslated region sequence of the retinal library oatp3 cDNA remains unpublished (1). The oatp3 expression pattern differences could also be explained by differences in the rat strains used in the two studies.

The high degree of sequence identity, particularly in the 3'-untranslated region, argues that the novel mouse cDNA in this study is orthologous to rat oatp3 and human OATP-A. The assignment of mouse and rat oatp3 as the rodent orthologs of human OATP-A was further supported by localization of the mouse oatp3 to a region of chromosome 6 syntenic with human chromosome 12p12 (28, 30). Kullak-Ublick et al. (30) showed by Northern blot analysis that OATP-A, like oatp3, is expressed at relatively high levels in brain and lung. Lower levels detected in the kidney and liver may represent cross-hybridization of the coding region probe with other members of the family or species differences. No OATP-A RNA was detected in small intestine. However, the low levels of oatp3 measured in rat intestine by RPA may be below the level of detection by Northern blot analysis. The uptake values of only six substrates are available for rat oatp3 and human OATP-A. Both proteins mediate transport of cholate, taurocholate, and tauroursodeoxycholate, although the $K_m$ of cholate varies greatly for the two transporters (8.8 $\mu$M for oatp3 and 93 $\mu$M for OATP-A). The cyanobacterial toxin microcystin is transported by OATP-A (14) but not by rat oatp3, and neither protein transports digoxin or methotrexate (35). The different transport properties may represent real species differences or differences between experimental systems.

It is widely accepted that active Na$^+$-dependent transport is the predominant mechanism for intestinal bile acid absorption and maintenance of the enterohepatic circulation under normal physiological conditions (20). However, the question of what fraction of the bile acid pool is absorbed by mechanisms other than the ASBT has not been fully resolved in humans or in experimental animal models. The identification of a second apical intestinal carrier for bile acids provides a molecular mechanism for the non-ASBT-mediated absorption and has important physiological and therapeutic implications. In pathophysiological states in which ASBT function is compromised, such as ileal resection (9), primary bile acid malabsorption (36), and ileal inflammation (44), induction of oatp3 may slow bile acid loss. In contrast, induction of oatp3-mediated intestinal bile acid absorption would compromise the efficiency of specific ASBT inhibitors being developed to treat hypercholesterolemia (27). Identification of the oatp3 in small intestine will facilitate studies, including knockout mouse models, to define the quantitative significance of this pathway for the absorption of bile acids, other nutrients, and drugs under physiological and pathophysiological conditions.

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The nucleotide sequences reported in this study have been submitted to the GenBank database under GenBank accession nos. AF083469 for rat oatp3 and AF240694 for mouse oatp3. The mouse sequence reported in this study has been submitted to the Mouse Genome Database (www.informatics.jax.org/support/nomen/) under the locus symbol Scl21a7.

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