

Isolation, Characterization and Differential Gene Expression of Multispecific Organic Anion Transporter 2 in Mice

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ABSTRACT

We isolated cDNA encoding a multispecific organic anion transporter 2 (OAT2) from the mouse kidney cDNA library. Isolated mouse OAT2 (mOAT2) consisted of 1623 base pairs that encoded a 540-amino acid residue protein with 12 putative membrane-spanning domains, and the amino acid sequence was 87% identical to that of rat OAT2 (rOAT2). The gene coding for mOAT2, *Slc22a7*, is found on chromosome 17C. Northern blot analysis revealed that the mOAT2 mRNA is abundantly expressed in the male mouse kidney, whereas it was predominantly expressed in both the liver and kidney of female mice. When expressed in *Xenopus laevis* oocytes, mOAT2 mediated the high affinity transport of glutarate ($K_m = 15.8 \pm 3.2 \mu\text{M}$) and

prostaglandin E_2 ($K_m = 5.2 \pm 0.5 \text{ nM}$) in a sodium-independent manner. mOAT2-expressing oocytes also mediated the uptake of α -ketoglutarate, glutarate, prostaglandin E_2 , *p*-aminohippuric acid, methotrexate, ochratoxin A, valproate, and allopurinol. However, we did not observe mOAT2-mediated uptake of salicylate. A wide range of structurally unrelated organic anions inhibited mOAT2-mediated glutarate uptake especially erythromycin, a potent inhibitor. These results indicate that isolated mOAT2 is a multispecific organic anion transporter having some differences in substrate specificity compared with rOAT2. In addition, we found that there exists a sex- and species-related differential gene expression of the OAT2 isoform.

Organic anion transporters play central roles in the elimination of a wide range of endogenous and exogenous anionic compounds including drugs, environmental toxicants, and their metabolites. Current extensive molecular studies have identified several families of multispecific organic anion transporters that are involved in the elimination of various organic anions (Moller and Sheikh, 1983; Boyer et al., 1992; Pritchard and Miller, 1993; Ullrich and Rumrich, 1993; Petzinger, 1994; Meier, 1995; Muller and Jansen, 1997; Ullrich, 1997). In the liver and kidney, water-soluble metabolites and organic anions are actively eliminated from the body via transporters across their basolateral and/or luminal membranes (Moller and Sheikh, 1983; Boyer et al., 1992; Pritchard and Miller, 1993; Ullrich and Rumrich, 1993; Petzinger, 1994; Meier, 1995; Bossuyt et al., 1996; Muller and Jansen, 1997; Ullrich, 1997). The proximal tubular cells of the kidney take up organic anions from the blood stream via

multispecific organic anion transport pathway(s) in the basolateral membranes (Moller and Sheikh, 1983; Ullrich and Rumrich, 1993; Pritchard and Miller, 1993; Ullrich, 1997). The liver is a prime metabolic organ and takes up various organic anions from the blood stream via various transporters located in the sinusoidal membrane (Petzinger, 1994; Meier, 1995; Muller and Jansen, 1997). Thus, the liver and the kidney play an important role in the elimination and excretion of organic compounds into urine and the gut.

Recently, organic anion transporter 1 (OAT1) belonging to the subgroup one of the solute carrier superfamily 22A has been isolated and determined to be located in the basolateral membrane in renal proximal tubules (Sekine et al., 1997; Sweet et al., 1997). OAT1 has a broad substrate specificity and interacts with a wide range of organic anions such as *p*-aminohippuric acid (PAH), dicarboxylates, cyclic nucleotides, prostaglandin E_2 (PGE_2) (Sekine et al., 1997; Sweet et al., 1997; Apiwattanakul et al., 1999). Subsequently, OAT1 orthologs have been isolated from humans and mice (Lopez-Nieto et al., 1997; Hosoyamada et al., 1999; Kuze et al., 1999; Lu et al., 1999; Race et al., 1999). So far, four additional

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ABBREVIATIONS: OAT, organic anion transporter; rOAT, rat organic anion transporter; mOAT, mouse organic anion transporter; hOAT, human organic anion transporter; PG, prostaglandin; NLT, novel liver-specific transport protein; MTX, methotrexate; PAH, *p*-aminohippuric acid; Roct, reduced in oc transporter; OTA, ochratoxin A; dCTP, deoxycytidine [5'- α -³²P]triphosphate; SSC, standard saline citrate; MES, 2-morpholinoethanesulfonic acid; GTC, guanidinium thiocyanate; FISH, fluorescent in situ hybridization; DAPI, 4,6-diamino-2-phenylindole; BSP, bromosulphothalein; ICG, indocyanine green; bp, base pair(s); kb, kilobase(s).

members belonging to the organic anion transporter family, OAT2 (Simonson et al., 1994; Sekine et al., 1998), OAT3 (Kusuhara et al., 1999; Race et al., 1999; Cha et al., 2001), OAT4 (Cha et al., 2000), and OAT5 (Sun et al., 2001), have been cloned and identified. Among these OAT homologs, rOAT2 is identical to the membrane protein previously called the novel liver-specific transport protein (NLT) (Simonson et al., 1994). NLT was initially isolated from a liver cDNA library using a monoclonal antibody raised against the glucagon receptor; rOAT2 was 42% identical to rat OAT1 (rOAT1) (Simonson et al., 1994; Sekine et al., 1998). We have demonstrated that rOAT2 mediates the transport of PGE₂, methotrexate (MTX), acetylsalicylate, PAH, and α -ketoglutarate in a sodium-independent manner (Sekine et al., 1998). Although several OAT isoforms have been isolated to date, information of the function of each isoform is still not sufficient. Moreover, the functional property of mouse OAT homologs is limited despite the fact that mouse OAT1 (mOAT1) (NKT) and mouse OAT3 (mOAT3) (Roct) isoforms have been isolated (Lopez-Nieto et al., 1997; Brady et al., 1999; Kuze et al., 1999). Among the OAT isoforms, only OAT2 is predominantly expressed in the liver, and this isoform is considered one of the key molecules in hepatic handling of organic anions.

Here, we describe the molecular cloning and functional characterization of mOAT2 from a mouse kidney cDNA library. Our results indicate that mOAT2 is a multispecific organic anion transporter with some differences in substrate specificity compared with rOAT2. Furthermore, the distribution of OAT2 shows sex- and species-related differences.

Experimental Procedures

Materials. [¹⁴C]glutarate (2.035 GBq/mmol), α -ketoglutarate (2.01 GBq/mmol), [³H]MTX (555 GBq/mmol), [³H]PGE₂ (7.429 TBq/mmol), [¹⁴C]salicylate (2.01 GBq/mmol) and [³H]valproate (2.019 GBq/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). [¹⁴C]PAH (1.50 GBq/mmol), [¹⁴C]allopurinol (51 GBq/mmol), and [³H]OTA (547.6 GBq/mmol) were purchased from Moravak Biochemicals (Brea, CA). Deoxycytidine [5'- α -³²P]triphosphate (dCTP) (111 TBq/mmol) was obtained from Muromachi Yakuhin Kaisha, LTD (Tokyo, Japan). All other chemicals not listed here were of the highest grade commercially available.

Construction of cDNA Library and Isolation of mOAT2. A nondirectional cDNA library for screening was prepared from mouse kidney poly(A)⁺ RNA using Superscript Choice System (Invitrogen, Carlsbad, CA) and was ligated into a phage vector λ ZipLox *Eco*RI arms (Invitrogen). The library was screened by homology using full-length rOAT2 cDNA labeled with [α -³²P]dCTP by random priming (T7Quick Prime Kit; Amersham Pharmacia Biotech, Uppsala, Sweden) as a probe (Feinberg and Vogelstein, 1983). Replicated filters of the phage library were hybridized overnight in a hybridization solution [50% formamide, 5 \times standard saline citrate (SSC), 3 \times Denhardt's solution, 0.2% SDS, 10% dextran sulfate, 0.3 μ g/ml denatured salmon sperm DNA, 2.5 mM sodium pyrophosphate, 25 mM MES, 0.03% Antifoam A, pH 6.5] at 37°C overnight. The filters were finally washed in 0.1 \times SSC and 0.1% SDS at 37°C. cDNA inserts in positive λ ZipLox phage were recovered in plasmid pZLI1 vector by in vitro excision.

Sequence Determination of mOAT2. Double-stranded cDNA of isolated clones were sequenced in both directions. Deleted clones obtained by a KiloSequence deletion kit (Takara, Tokyo, Japan) and specially synthesized oligonucleotide primers were used for sequencing of mOAT2 cDNA, which was sequenced by the dye terminator method using a dye primer cycle sequencing kit (Applied Biosystems,

Foster City, CA) and automated Applied Biosystems 310 DNA sequencer. The sequence was assembled and analyzed using DNASIS-Mac, version 3.6 (Hitachi Software Engineering, Yokohama, Kanagawa, Japan).

Total RNA Isolation. Total RNA was isolated from various tissues using the acid guanidinium thiocyanate (GTC)-phenol-chloroform extraction method as described by Chomczynski and Sacchi (1987). The tissue was homogenized in GTC solution (4.0 M GTC containing 25 mM sodium citrate, pH 7.0, 0.5% sarkosyl, and 0.1 M 2-mercaptoethanol; 10 ml of GTC solution/100 mg of liver tissue). The RNA was extracted twice into phenol and chloroform (1 volume of phenol/0.2 volumes of chloroform/1 volume of GTC solution) and precipitated with isopropanol at room temperature. The pellet was dissolved in 0.3 ml of GTC solution and precipitated with isopropanol at -20°C. The resulting RNA pellet was washed with ice-cold 80% ethanol and dissolved in an appropriate volume of diethylpyrocarbonate-treated water. The RNA yield, purity, and integrity were determined by the 260 nm/280 nm absorbance ratio (>1.6) and checked with 1.0% agarose/formamide gel.

Northern Blot Analysis. Two micrograms of poly(A)⁺ RNA prepared from various mouse tissues were loaded onto 1% agarose/formaldehyde gel. For the study of the sex-related differential gene expression, 20 μ g of total RNA obtained from the liver and kidney was loaded onto 1.0% agarose/formaldehyde gel. After electrophoresis, the nucleic acids were transferred onto a nylon membrane (Hybond N+; Amersham Biosciences, Uppsala, Sweden). The filter was hybridized at 42°C overnight in a hybridization solution (50% formamide) with a full-length cDNA of mOAT2 or β -actin, which were randomly labeled with [³²P]dCTP. The filter was finally washed in 0.1 \times SSC/0.1% SDS at 42°C.

Chromosomal Slide Preparation. Lymphocytes were isolated from mouse spleen and cultured at 37°C in an RPMI 1640 medium supplemented with 15% fetal calf serum, 3 μ g/ml concanavalin A, 10 μ g/ml lipopolysaccharide and 5 \times 10⁻⁵ M mercaptoethanol. After 44 h, the cultured lymphocytes were treated with 0.18 mg/ml bromodeoxyuridine for an additional 14 h. The synchronized cells were washed and recultured at 37°C for 4 h in α -minimal essential medium with thymidine (2.5 μ g/ml). Chromosome slides were made by the conventional methods used for human chromosome preparation (hypotonic treatment, fixation, and air dry).

Probe Labeling and Fluorescent in Situ Hybridization. The mOAT2 DNA probe was biotinylated with dATP using a Invitrogen BioNick labeling kit (15°C, 1 h) (Heng et al., 1992). The procedure for fluorescent in situ hybridization (FISH) detection was performed according to Heng et al. (1992) and Heng and Tsui (1993). Briefly, slides were heated at 55°C for 1 h. After RNase A treatment, the slides were denatured in 70% formamide in 2 \times SSC for 2 min at 70°C followed by dehydration with ethanol. Probes were denatured at 75°C for 5 min in a hybridization mix consisting of 50% formamide and 10% dextran sulfate and prehybridized for 15 min at 37°C. Probes were added to the denatured samples on the slides. After overnight hybridization, slides were amplified by the method of Heng et al. (1992). FISH signals and the 4,6-diamino-2-phenylindole (DAPI) banding pattern were recorded separately by taking photographs, and the assignment of the FISH mapping data with chromosomal bands was achieved by superimposing FISH signals with DAPI-banded chromosomes (Heng and Tsui, 1993).

Xenopus Laevis Oocyte Preparation, cRNA Synthesis, and Functional Expression of Transport Activity. Isolation of *X. laevis* oocytes was performed as described elsewhere (Sekine et al., 1997). Stage V and VI defolliculated oocytes were selected throughout this experiment. To remove the follicular layer from *X. laevis* oocytes, collagenase A (Roche Applied Science, Mannheim, Germany) was used at a final concentration of 2.0 mg/ml in OR2 solution (83 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES, pH 7.5) and slowly shaken for 3 h at room temperature. The isolated cDNA, mOAT2, was linearized with *Bam*HI, and the capped cRNA was transcribed in vitro by T7 RNA polymerase (Sekine et al., 1997).

Defolliculated oocytes were microinjected with 10 ng of in vitro transcribed cRNA and incubated for 3 days in a modified Barth's solution containing 50 µg/ml gentamicin at 18°C. Uptake experiments of radiolabeled substrates, as indicated in each experiment, were performed in ND 96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH 7.4) at room temperature. Oocytes were incubated in 450 µl of the same solution containing radiolabeled substrates for 1 h at room temperature. The uptake was terminated by the addition of 2 ml of ice-cold ND 96 solution, and the oocytes were washed with the same solution at least five times. The oocytes were solubilized with 10% SDS, and accumulated radioactivity was determined by a liquid scintillation counter. The experiments were repeated with oocytes from at least five frogs.

Kinetic Study. Concentration-dependent uptake experiments of PGE₂ and glutarate via mOAT2 were performed with each compound at a final concentration range of 1, 5, 10, 25, and 50 nM and 1, 2, 5, 10, 50, and 100 µM, respectively. The compounds were incubated with expressing mOAT2 oocytes for 1 h at room temperature, stopped with ice-cold ND96 solution and washed five times as described above. Individual oocytes were transferred to scintillation vials and dissolved in 250 µl of 10% SDS. A scintillation cocktail was added, and radioactivity was counted. Counts in control uninjected oocytes were subtracted from the counts in cRNA-injected oocytes. Data are presented as mean ± S.E.M., except for kinetic constants, for which the error represents the error of the fit. K_m indicates the Michaelis-Menten constant (nano- or micromolar).

Inhibition Study. For the inhibition experiments, oocytes expressing mOAT2 were incubated for 1 h in ND96 solution containing 5 µM [¹⁴C]glutarate in the presence or absence of various inhibitors at a final concentration of 1 mM, except for bromosulphophthalein (BSP) (250 µM). Cimetidine, indocyanine green (ICG), cefoperazone, enalapril, oxaloacetate, and erythromycin were directly dissolved in ND96 solution from stock solutions. These stock solutions of the inhibitors were prepared in dimethyl sulfoxide and diluted to a final concentration as described above. The final concentration of dimethyl sulfoxide in the assay medium did not exceed 1.0%.

Statistical Analysis. Kinetic data from experiments measuring the uptake of radiolabeled substrates were fit to the Michaelis-Menten equation by nonlinear least-squares regression analysis with standard errors derived from these curves. Comparisons of data measuring initial rates of uptake of radiolabeled substrates in the

presence and absence of inhibitors were performed by the unpaired Student's *t* test.

Results

Cloning of mOAT2. A nondirectional cDNA library of mouse kidney was screened using a full-length rOAT2 cDNA as a probe. We finally purified seven positive plaques. mOAT2 has a single open reading frame of 1623 bp encoding a 540-amino acid sequence (GenBank/EBI, AB069965) (Fig. 1). Kyte-Doolittle hydropathy analysis with a window size of 19 predicts that mOAT2 has 12 putative transmembrane domains. There are three consensus sequences for *N*-glycosylation sites (Asn⁵⁷, Asn⁹¹, and Asn³⁵⁶) that are predicted to be in the first hydrophilic loop and between the seventh and eighth transmembrane domains in this protein. The sequence also contains six potential protein kinase C-dependent phosphorylation sites (Ser¹⁶⁴, Thr¹⁹⁸, Ser²⁵⁴, Ser²⁷⁹, Ser³²⁷, and Thr⁵¹³) located in intracellular loops. The amino acid sequence alignments of mOAT2 compared with rOAT2 is also shown in Fig. 1. The two sequences are 88% identical. The amino acid sequence of mOAT2 is also 41% identical to rOAT1 (Sekine et al., 1997), 40% for mOAT1 (NKT) (Lopez-Nieto et al., 1997), 42% for hOAT1 (Hosoyamada et al., 1999), 40% for rOAT3 (Kusuhara et al., 1999), 41% for mOAT3 (Roct) (Brady et al., 1999), 39% for hOAT3 (Cha et al., 2001) and 38% for hOAT4 (Cha et al., 2000). While this study was being conducted, a sequence from the mouse kidney with an unknown function was submitted to GenBank (BC013474). The nucleotide sequence of mOAT2 is 99% identical to that of this gene (BC013474) except one base change (G at position 136 to A) resulting in the change of an amino acid (Ala⁴⁶ to Thr).

Tissue Distribution of mOAT2 mRNA. We have found that rOAT1, rOAT3, hOAT3, and hOAT4 mRNAs are expressed predominantly in the kidney (Sekine et al., 1997; Kusuhara et al., 1999; Cha et al., 2000, 2001). In contrast,

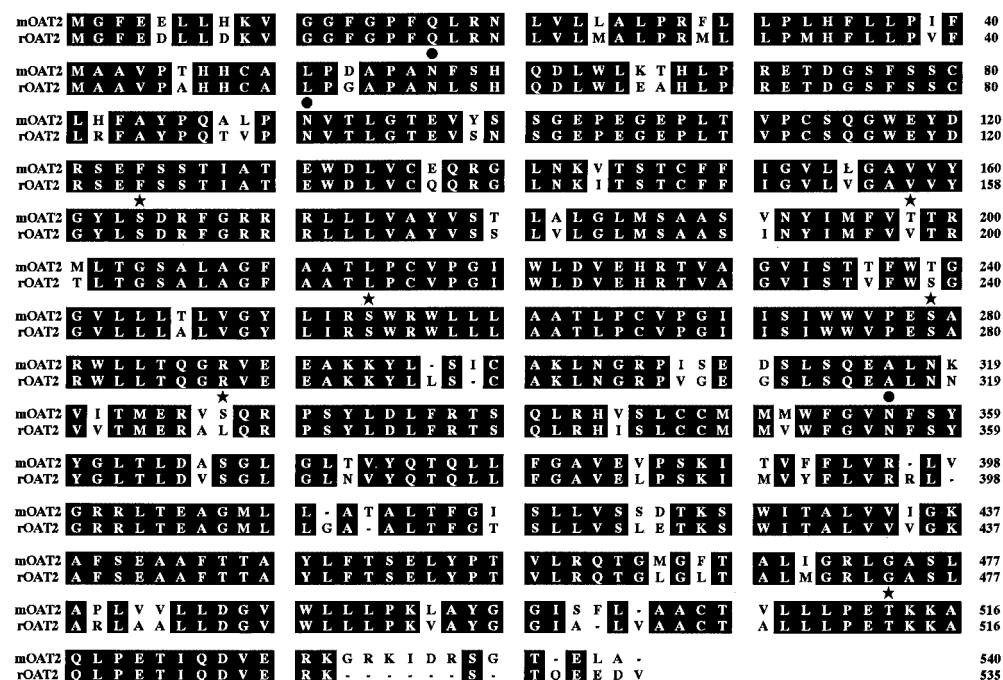


Fig. 1. Deduced amino acid sequence of mOAT2 aligned with that of rOAT2. Boxed residues indicate conserved residues in two transporters. ●, putative *N*-glycosylation sites; ★, putative protein kinase C-dependent phosphorylation sites.

rOAT2 mRNA was strongly expressed in the liver and relatively weakly expressed in the kidney (Simonson et al., 1994; Sekine et al., 1998). Thus, the expression level of individual OAT isoforms depends on the organ in which they are found. We subsequently examined, based on these findings, the tissue distribution of mRNA coding for mOAT2. Northern blot analysis of the mOAT2 revealed that a single mRNA of 2.4 kb is abundant in the male mouse kidney and faintly expressed in the liver (Fig. 2A). Hybridization signals could not be detected in mRNA isolated from other tissues, such as the skeletal muscle, testis, pancreas, heart, brain, lung, and eye. Thus, the expression of this gene is tissue-specific. The result indicates that there exists a species-related differential distribution of the *OAT2* gene expression. The result of Northern blot analysis regarding sex differences of the *mOAT2* gene is shown in Fig. 2B. The mOAT2 mRNA was clearly expressed in the liver of female mice but weakly expressed in male mice (Fig. 2B). No gender-related differential gene expression of mOAT2 was detected in other tissues, such as skeletal muscle, testis, pancreas, heart, brain, lung, and eye (data not shown). Taken together, our results indicate that there exists a sex- and species-related differential expression of OAT2 isoform in rodents.

Chromosomal Localization of mOAT2. To determine the chromosomal localization of the *mOAT2* gene, we subsequently performed a FISH analysis. Under the conditions described in the experimental procedures, FISH detection efficiency was 88% using mOAT2 cDNA as a probe (among 100 checked mitotic figures, 88 of them showed hybridization signals on one pair of chromosomes). Because the DAPI banding was performed to identify the specific chromosome, the assignment between signals from the probe and mouse chromosome 17 was obtained. The detailed position was further determined based on a summary from 10 photos (Fig. 3). Therefore, this gene is mapped to chromosome 17C.

Functional Characterization of mOAT2. Various endogenous and exogenous organic anion substrates such as PAH, nonsteroidal anti-inflammatory drugs, diuretics, angiotensin-converting enzyme inhibitor, conjugated steroid hormones, and intermediates of the tricarboxylic acid cycle (dicarboxylate) are transported by OAT1, -3, and -4 (Sekine et al., 1997; Apiwattanakul et al., 1999; Hosoyamada et al., 1999; Kusuhara et al., 1999; Cha et al., 2000, 2001). Liver-predominant rOAT2 mediates the transport of salicylate, PGE₂, MTX, acetylsalicylate, PAH, and α -ketoglutarate (Sekine et al., 1998). Thus, all OAT isoforms are known to be polyspecific organic anion transporters. We chose, based on these findings, some of these organic anions to test substrate specificity of mOAT2. *X. laevis* oocytes injected with mOAT2 cRNA were used for the transport characterization. As shown in Fig. 4, mOAT2 mediated the transport of [¹⁴C] α -ketoglutarate, [³H]MTX, [³H]PGE₂, [¹⁴C]valproate, [¹⁴C]glutarate, [³H]OTA, [¹⁴C]PAH, and [³H]allopurinol. We did not observe mOAT2-mediated transport of [³H]estradiol glucuronide, [³H]estrone sulfate, [¹⁴C]indomethacin, [¹⁴C]3'-azido-3'-deoxythymidine, [¹⁴C]testosterone, [¹⁴C]progesterone, and [¹⁴C]androstenedione (data not shown). Interestingly, mOAT2 also failed to transport [¹⁴C]salicylate, despite the fact that it is a good compound for rOAT2 (Fig. 4) (Sekine et al., 1998). These findings indicate that mOAT2 is a multispecific organic anion transporter with some differences in substrate specificity compared with the rat ortholog.

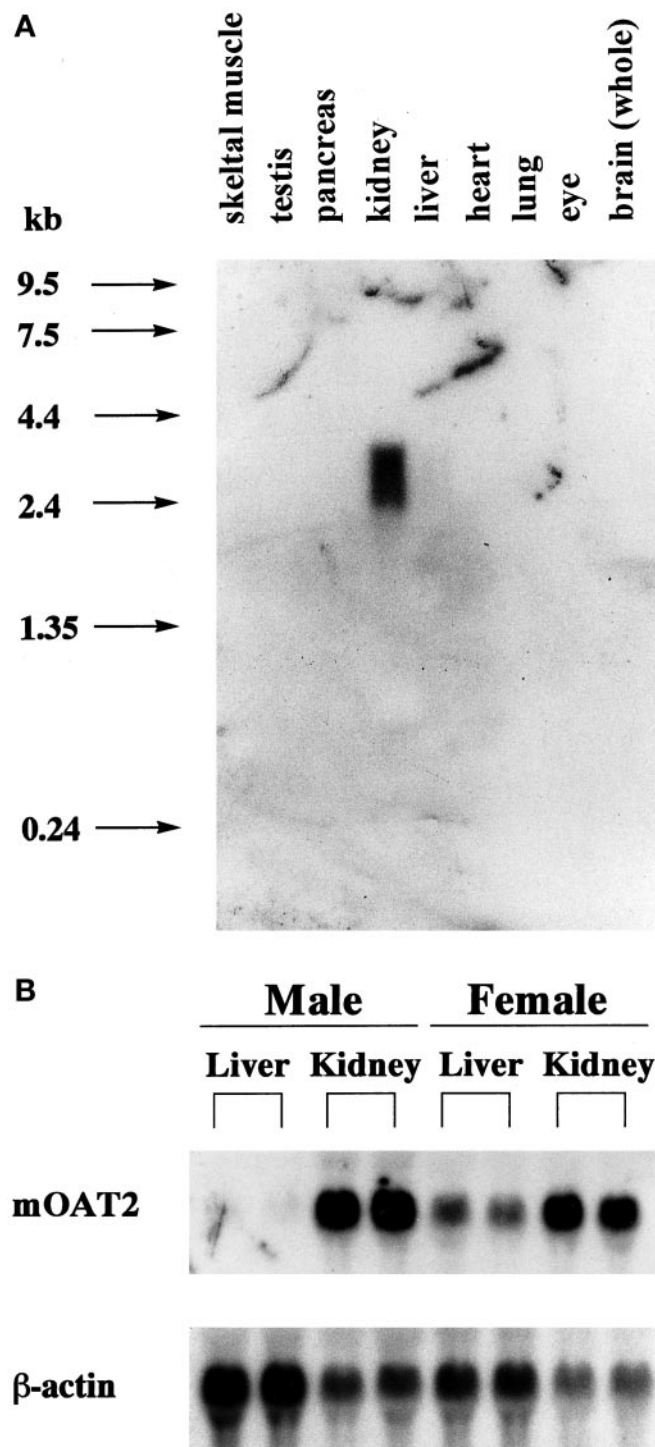


Fig. 2. Localization and tissue distribution of mOAT2 mRNA by Northern blot analysis. A, a high-stringency Northern blot analysis of poly(A)⁺ RNA from various tissues of male mouse probed with ³²P-labeled mOAT2 cDNA. Poly(A)⁺ RNA (2 μ g) was loaded onto 1.0% agarose/formamide gel and transferred to a nylon membrane. A highly expressed 2.4-kb transcript was detected predominantly in the kidney and relatively weakly expressed in the liver. B, a high-stringency Northern blot analysis in the liver and kidney of male and female mice. Total RNA (20 μ g) isolated from mice liver and kidney was loaded onto 1.0% agarose/formamide gel. A highly expressed 2.4-kb transcript was detected in kidneys of male and female mice and the in the liver of female mice, whereas a faint positive signal was detected in the male liver. ³²P-labeled β -actin cDNA was used for the control. Other experimental conditions and details are described under *Experimental Procedures*.

Given the high sequence identity between rOAT2 and mOAT2, we assumed that the function of both proteins is very similar. The transport of compounds mediated by rOAT2 is sodium-independent (Sekine et al., 1998). Therefore, we next examined whether the uptake of organic anions

mediated by mOAT2 is also sodium-independent. The uptake of [14 C]glutarate via mOAT2 was not affected by the replacement of extracellular sodium with lithium (Fig. 5). Additionally, replacing the extracellular sodium with choline did not inhibit the mOAT2-mediated uptake of [14 C]glutarate (data

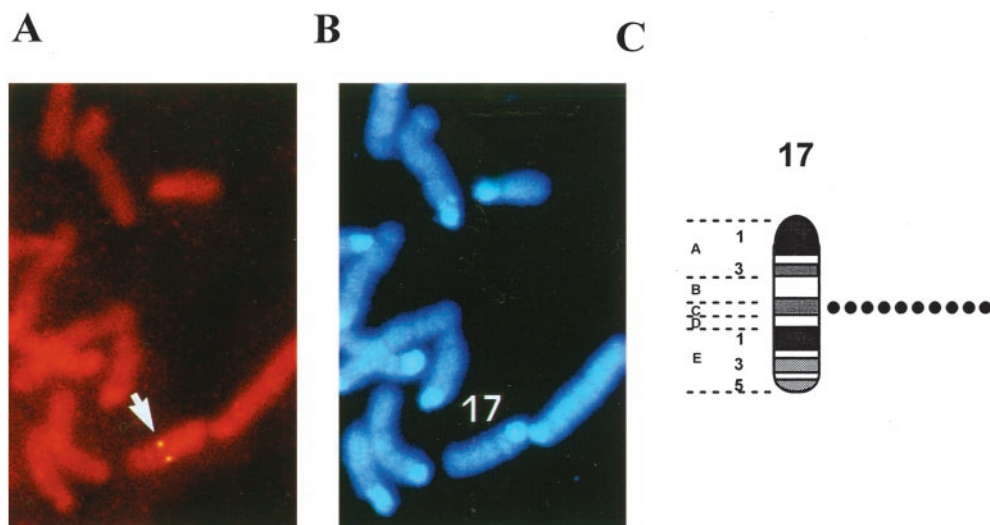


Fig. 3. Diagram of FISH mapping results for probe mOAT2. A, the FISH signals on mouse chromosome. B, the same mitotic figure stained with DAPI to identify mouse chromosome 17. C, diagram of FISH mapping results for probe mOAT2. Each dot represents the double FISH signals detected on mouse chromosome 17, region C. Other experimental conditions and details are described under *Experimental Procedures*.

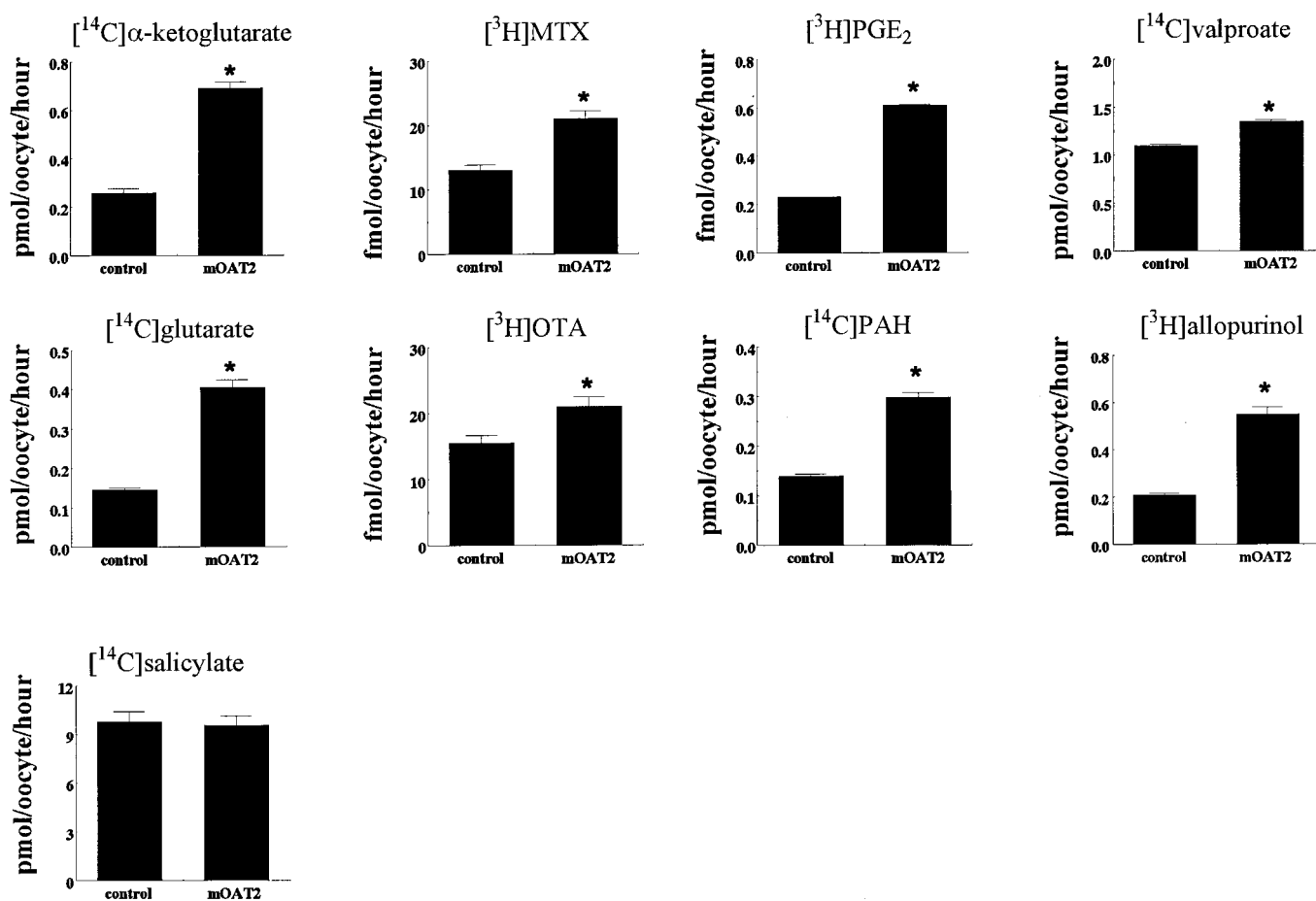


Fig. 4. Uptake of various 3 H- or 14 C-labeled compounds by mOAT2-expressing oocytes. The uptake rates of radiolabeled compounds ([14 C]α-ketoglutarate, 5 μ M; [3 H]MTX, 0.5 μ M; [3 H]PGE₂, 5 nM; [14 C]valproate, 5 μ M; [14 C]glutarate, 5 μ M; [3 H]OTA, 5 μ M; [14 C]PAH, 5 μ M; [3 H]allopurinol, 5 μ M; [14 C]salicylate, 20 μ M) by the control or mOAT2 expressed oocytes were measured for 1 h. Values are mean \pm S.E.M. of 8–15-oocyte determinations. Other experimental conditions and details are described under *Experimental Procedures*. The significance between control (water-injected) and mOAT2-cRNA-injected oocytes was determined by the unpaired *t* test (*, *p* < 0.01).

not shown). We conclude that the transport of organic compounds mediated by mOAT2, as well as rOAT2, is sodium independent.

The concentration dependence of the uptake of [14 C]glutamate and [3 H]PGE₂ via mOAT2 is shown in Fig. 6. The mOAT2-mediated uptake of these two compounds showed saturable kinetics and could be modeled by the Michaelis-Menten equation. Nonlinear regression analyses yielded K_m values of $15.8 \pm 3.2 \mu\text{M}$ and $5.2 \pm 0.5 \text{ nM}$ for glutamate and PGE₂, respectively (Fig. 6, A and B).

Inhibition Study. To investigate further the substrate specificity of mOAT2, we subsequently examined the inhibition of mOAT2-mediated [14 C]glutamate uptake by various compounds. As illustrated in Fig. 7, *cis*-inhibitory effects were observed for structurally unrelated organic anions. The inhibition of the mOAT2-mediated transport of [14 C]glutamate exhibited a rank order of erythromycin > BSP > cimetidine = ICG = enalapril > cefoperazone > oxaloacetate (Fig. 7). Although erythromycin did not inhibit the rOAT2-mediated uptake of [14 C]salicylate (Sekine et al., 1998), this compound proved to be the most potent inhibitor for the mOAT2-mediated uptake of [14 C]glutamate. These results suggest that substrate specificity of mOAT2 is different from that of rOAT2.

Discussion

In the present study, we report the isolation and functional characterization of multispecific OAT2 from a mouse kidney cDNA library. mOAT2 has an open reading frame encoding a 540 amino acid protein, and the hydropathy plot of mOAT2 using the algorithm of Kyte-Doolittle predicts 12 putative membrane-spanning domains. The *Slc22a7* gene, coding for mOAT2, is located on chromosome 17C. The *mOAT2* gene was abundantly expressed in the male mouse kidney but faintly expressed in the liver, whereas the expression level of

this gene was detected in almost equal amounts in the female liver and kidney. Moreover, we found that the expression of mOAT2 in the liver of rats and mice is quite different, indi-

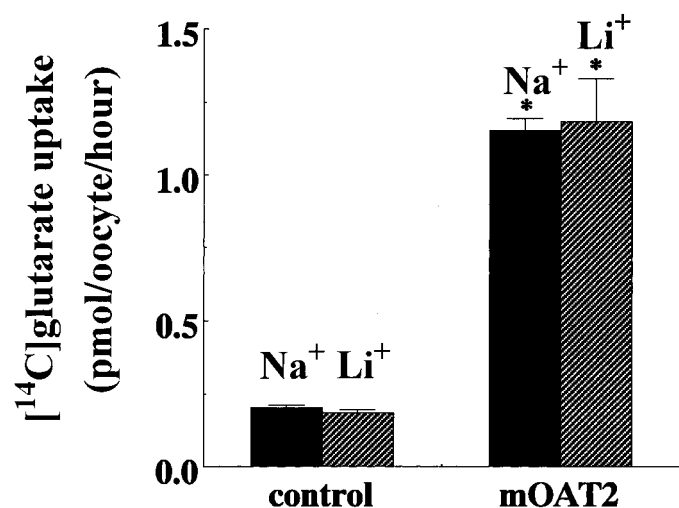


Fig. 5. Effect of extracellular cation on [14 C]glutamate uptake in *X. laevis* oocytes expressing mOAT2. The tracer concentration used was $50 \mu\text{M}$. The uptake rate of [14 C]glutamate by control (water-injected) oocytes or mOAT2-expressing oocytes for 1 h was measured at room temperature in the presence (■) or absence (▨) of extracellular Na⁺. Extracellular Na⁺ was replaced with an equimolar concentration of lithium. Values are mean \pm S.E.M. of 5–10-oocyte determinations. The significance between control (water-injected) and mOAT2-cRNA-injected oocytes was determined by the unpaired *t* test (*, $p < 0.001$). Other experimental conditions and details are described under *Experimental Procedures*.

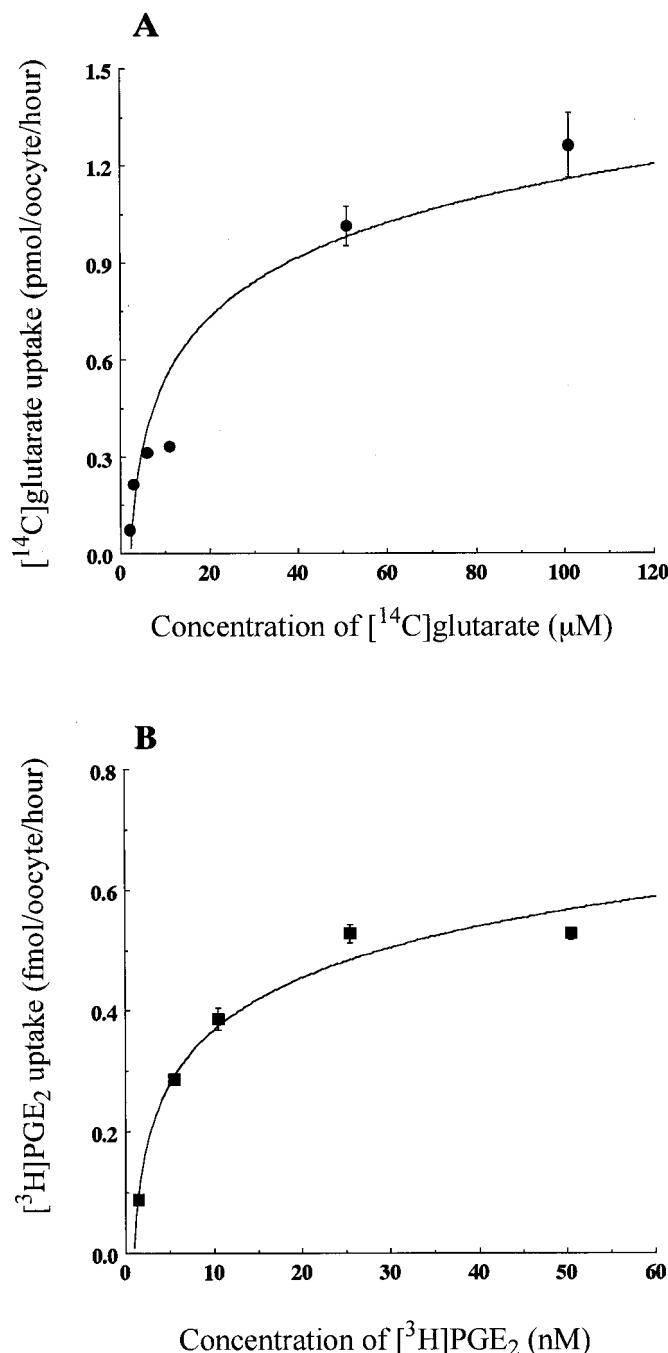


Fig. 6. Concentration dependence of mOAT2-mediated [14 C]glutamate (A) and [3 H] prostaglandin E₂ (B). The uptake experiments of glutamate and prostaglandin E₂ via mOAT2 were performed with each compound at final concentration ranges of 1, 2, 5, 10, 50, and 100 μM and 1, 5, 10, 25, and 50 nM, respectively. The uptake rates of two compounds by the control (water-injected) or mOAT2-expressing oocytes for 1 h were measured at variable concentrations. The uptake was saturable with K_m values of $15.8 \pm 3.2 \mu\text{M}$ and $5.2 \pm 0.5 \text{ nM}$ for glutamate and PGE₂, respectively, and fit to the Michaelis-Menten curve. Values are mean \pm S.E.M. of 5–14-oocyte determinations. The mOAT2-mediated transport was determined by subtracting the transport velocity in control (water-injected) oocytes from that in mOAT2-expressing oocytes. Other experimental conditions and details are described under *Experimental Procedures*.

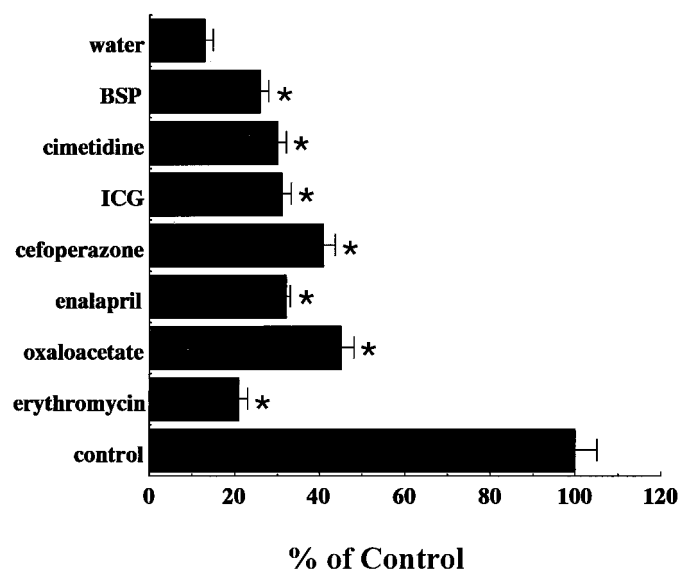


Fig. 7. Inhibition of mOAT2-mediated [^{14}C]glutamate uptake by various compounds. The uptake rate of [^{14}C]glutamate by mOAT2-expressing oocytes or control (water-injected) oocytes was determined in the presence or absence of 1 mM except for BSP (250 μM). The concentration of [^{14}C]glutamate was 5 μM . The values were expressed as a percentage of [^{14}C]glutamate uptake in mOAT2-expressing oocytes in the absence of the inhibitor. Values are mean \pm S.E.M. of 4–7-oocyte determinations. The significance between control (water-injected) and mOAT2-cRNA-injected oocytes was determined by the unpaired t test (*, $p < 0.01$). Other experimental conditions and details are described under *Experimental Procedures*.

cating that there is a species- and sex-dependent expression of OAT2. Functional experiments have revealed that mOAT2 mediates the uptake of various organic anions such as α -ketoglutarate, MTX, PGE_2 , valproate, glutarate, OTA, PAH, and allopurinol, but not salicylate, in a sodium-independent manner. The concentration dependence of the uptake of glutarate and PGE_2 via mOAT2 was saturable with K_m values of 15.8 ± 3.2 μM and 5.2 ± 0.5 nM for glutarate and PGE_2 , respectively. The mOAT2-mediated transport of glutarate was strongly inhibited by various organic compounds, erythromycin in particular. All of these findings together with previous reports (Sekine et al., 1997; Kusuha et al., 1999; Cha et al., 2000, 2001) suggest that mOAT2 is a multispecific organic anion transporter.

To date, at least five distinct OAT isoforms have been identified and their transport characteristics have already been characterized, except for OAT5 (Sekine et al., 1997, 1998; Kusuha et al., 1999; Cha et al., 2000; Sun et al., 2001). These isoforms are multispecific transporters that interact with a wide range of organic anions such as nonsteroidal anti-inflammatory drugs, diuretics, and β -lactam antibiotics (Sekine et al., 1997, 1998; Kusuha et al., 1999; Cha et al., 2000; Sun et al., 2001). Of the prototype substrate for individual OAT isoforms, we have shown that rOAT1 mediates the high-affinity transport of PAH (Sekine et al., 1997). Sulfate-conjugated steroids (estrone sulfate and dehydroepiandrosterone sulfate) and salicylate are preferred substrates of hOAT4 and rOAT2, respectively, although a very low rate of uptake of PAH via OAT2/OAT4 has also been observed (Sekine et al., 1998; Cha et al., 2000). OAT3, as well as OAT1, is predominantly expressed in the kidney and mediates the transport of PAH (Cha et al., 2001). Thus, substrates for the individual OAT isoforms partially overlap, but

it seems to be the same compound in different species. For example, hOAT1 also mediated the transport of PAH as well as rat and mouse OAT1 (Hosoyamada et al., 1999; Pavlova et al., 2000). However, we did not observe the mOAT2-mediated uptake of salicylate, despite the fact that salicylate is a good substrate of rOAT2 (Sekine et al., 1998). Our results indicate that the mOAT2 transporter has somewhat different substrate specificity than that of rOAT2.

Based on the transport experiment mediated by mOAT2, we subsequently studied the inhibitory effect of the mOAT2-mediated uptake by various organic anions to obtain more detailed substrate selectivity. Because the chemical structure between glutarate and α -ketoglutarate is very similar, we chose glutarate for this experiment. The transport of glutarate mediated by mOAT2 interacted with structurally unrelated compounds such as BSP, ICG, and cefoperazone as observed in rOAT2 (Sekine et al., 1998). Specifically, we observed that erythromycin is the most potent inhibitor in oocytes expressing mOAT2 compared with rOAT2 indicating that these closely-related proteins have differences in functions. At present, however, we have no definite explanations for the differential functions between rOAT2 and mOAT2; it is possible that the binding interaction between the substrate and the carrier may vary because the molecular size and lipophilicity of salicylate is larger than that of glutarate. Comparison of the transport profile of glutarate and salicylate mediated by the OAT2 isoform would also lead to further information of substrate specificity. In this respect, further precise studies (i.e., structure-function analysis) are needed to determine whether erythromycin is able to inhibit transport of glutarate uptake mediated by rOAT2.

It has been reported that rOAT1 and rOAT3 are predominantly expressed in the kidney (Sekine et al., 1997; Kusuha et al., 1999), whereas rOAT2 (NLT) is abundantly expressed in the liver (Simonson et al., 1994; Sekine et al., 1998). OAT4 is also represented in the human placenta as well as in the kidney (Cha et al., 2000). Thus, the expression level of the individual OAT isoform depends on the organ in which the isoform is found. In the present study, we found that mOAT2 mRNA in male mice is predominantly expressed in the kidney but faintly expressed in the liver, whereas this gene is markedly expressed in the female liver and kidney. On this basis, we examined whether there exists such a sex-related differential expression of mOAT1 (NKT) and mOAT3 (Roc) genes. We observed that they are only kidney and no sex differences (data not shown), indicating that only mOAT2 shows a sex-related differential distribution among these OAT isoforms. Although a previous study showed a rather broad distribution of the mOAT2 (NLT) isoform in mice (Pavlova et al., 2000), the study was done using human cDNA encoding part of the rOAT2 probe which could produce nonspecific hybridization signals. From all of these findings, we concluded that only the mOAT2 isoform is expressed with sex dependence in the liver. Whether such a sex-dependent expression of mOAT2 could be observed at protein levels needs further study.

A similar observation of the sex-related differential gene expression in the liver and/or kidney has been reported. For example, the male-liver specific cytochrome P450 species CYP2C11 is expressed only in the liver, whereas CYP2C12 (female liver-specific) is detected only in females (Gonzalez, 1989). These sex-specific cytochrome P450 isoforms can be

regulated by the secretion pattern of growth hormones (Kamataki et al., 1983; Yamazoe et al., 1989; Imaoka et al., 1992). Taking these facts into consideration, observed sex differences in the expression of the OAT2 isoform of the liver may be regulated by sex steroids, such as testosterone, resulting in the sex-dependent expression of the OAT2 isoform. Taken together, these data lead us to conclude that there is a sex- and species-related differential expression of OAT2 mRNA in rodents. For a comprehensive understanding of the physiological role of the OAT2 isoform, investigation of the contribution of OAT2 in the liver is required from a pharmacological and pharmacokinetic point of view. It would be of interest to elucidate whether there is such a sex-associated differential distribution of the OAT isoform in other species such as the rat and the human.

In conclusion, we describe the molecular cloning, functional characterization, and sex-related differential gene expression of OAT2 in mice. We found that the expression level of mOAT2 mRNA in the liver and kidney of male mice is different from that of female mice. Uptake experiments have revealed that mOAT2 mediates the transport of various kinds of structurally unrelated drugs and chemicals in a sodium-independent manner. The mOAT2-mediated transport of glutarate was strongly inhibited by the addition of erythromycin *in vitro*. The mOAT2 transporter has somewhat different substrate specificity than rOAT2. Our results explain the sex-associated differences in pharmacokinetics and toxicokinetics of drugs and chemicals *in vivo* and indicate that rats and mice are good tools for investigation of drug development and construction of an organ and/or a transporter targeting drug-delivery system. Hence, attention should be paid to the sex and species of experimental animals when testing anionic drugs and chemicals in pharmacokinetic and toxicokinetic studies.

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