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Identification and Characterization of Human Organic Anion Transporter 3 Expressing Predominantly in the Kidney

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ABSTRACT

A cDNA encoding a multispecific organic anion transporter 3 (hOAT3) was isolated from a human kidney cDNA library. The hOAT3 cDNA consisted of 2179 base pairs that encoded a 543-amino-acid residue protein with 12 putative transmembrane domains. The deduced amino acid sequence of hOAT3 showed 36 to 51% identity to those of other members of the OAT family. Northern blot analysis revealed that hOAT3 mRNA is expressed in the kidney, brain, and skeletal muscle. When expressed in *Xenopus laevis* oocytes, hOAT3 mediated the transport of estrone sulfate (K_m = 3.1 μM), *p*-aminohippurate (K_m = 87.2 μM), methotrexate (K_m = 10.9 μM), and cimetidine (K_m = 57.4 μM) in a sodium-independent manner. hOAT3 also mediated the transport of dehydroepiandrosterone sulfate, ochratoxin A, PGE_2, estradiol glucuronide, taurocholate, glutarate, cAMP and uric acid. Estrone sulfate did not show any *trans*-stimulatory effects on either influx or efflux of [^{3}H]estrone sulfate via hOAT3. hOAT3 interacted with chemically heterogeneous anionic compounds, such as nonsteroidal anti-inflammatory drugs, diuretics, sulfobromophthalein, penicillin G, bile salts and tetraethyl ammonium bromide. The hOAT3 protein was shown to be localized in the basolateral membrane of renal proximal tubules and the hOAT3 gene was determined to be located on the human chromosome 11q12-q13.3 by fluorescent in situ hybridization analysis. These results suggest an important role of hOAT3 in the excretion/detoxification of endogenous and exogenous organic anions in the kidney.

The kidney, as well as the liver, plays a primary role in the excretion of drugs and drug metabolites (Moller and Sheikh, 1982; Pritchard and Miller, 1993; Ullrich and Rumrich, 1993; Meier, 1995; Muller and Jansen, 1997). In addition to glomerular filtration, the kidney excretes charged drugs via carrier-mediated pathways, which are organic anion and organic cation transport pathways, in renal proximal tubular cells (Sperber, 1959; Weiner and Mudge, 1964; Ullrich and Rumrich, 1988; Pritchard and Miller, 1991). In particular, the organic anion transport pathway has been shown to mediate the elimination of various drugs.

Transepithelial transport of organic anions in proximal tubules is carried out by two distinct transporters; first, organic anions are transported from the peritubular plasma by basolateral organic anion transporter(s) and subsequently effluxed into the tubular lumen by luminal transporter(s). Until early 1990s, renal organic anion transport was thought to be carried out by a few carrier proteins that showed wide substrate specificity. Various anionic drugs have been indicated to be taken up into the proximal tubular cells by the classic *p*-aminohippurate (PAH) transporter. For example, β-lactam antibiotics were suggested to be rapidly extracted by the kidney via the PAH transporter, and the concomitant use of probenecid, which is a typical inhibitor of the system reduces renal elimination of β-lactams (Ullrich et al., 1989). Recently, we have cloned the classic PAH transporter expressed in the basolateral membranes of proximal tubular cells from the rat kidney, and designated it as rat organic anion transporter 1 (rOAT1) (Sekine et al., 1997). OAT1 is a PAH/dicarboxylate exchanger and mediates the high-affinity transport of PAH in a sodium-independent manner. Analysis of heterologous expression systems of rOAT1 in oocytes and culture cells revealed that rOAT1 has the ability to transport anionic drugs, such as β-lactam antibiotics, NSAIDs, methotrexate, and antiviral drugs, as well as various endogenous

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The nucleotide sequence reported in this article has been submitted to the GenBank/EBI Data bank with accession number AB042505.

ABBREVIATIONS: hOAT, human organic anion transporter; PAH, *para*-aminohippurate; rOAT, rat organic anion transporter; DHEA, dehydroepiandrosterone; PG, prostaglandin; TEA, tetraethylammonium; PCR, polymerase chain reaction; EST, expressed sequence tag; SSC, standard saline citrate; FISH, fluorescent in situ hybridization; MES, 4-morpholineethanesulfonic acid; MTX, methotrexate; DAPI, 4,6-diamidino-2-phenylindole; oatp, organic anion transporting polypeptide; Mrp, multidrug resistance associated protein.

organic anions and exogenous substances. The transport properties of rOAT1 are nearly identical to those of the classic PAH transporter. In addition, we have identified several OAT isoforms: rOAT2 (Sekine et al., 1998), rOAT3 (Kusuhara et al., 1999), and hOAT4 (Cha et al., 2000). All of these isoforms are commonly expressed in the kidney, and their potential roles in renal handling of organic anions have been indicated.

Although the number of the OAT isoforms is growing rapidly, characterization of each isoform is very limited. Moreover, information on human homologs is sparse. Among the OAT isoforms, rOAT3, as well as rOAT1, exhibits a markedly wide substrate selectivity, and its human homolog is considered a key molecule in the renal handling of organic anions. Because hOAT1 has been suggested to show rather limited capacity for organic anion transport, hOAT3 might play a large role in the human kidney.

Here, we report on the molecular cloning, functional characterization, and localization of hOAT3. The results indicate that hOAT3 shows a very wide substrate specificity and is localized in basolateral membranes of proximal tubular cells.

Experimental Procedures

Materials. The materials used were purchased from following sources: [^{14}C]*p*-aminohippurate (2.00 GBq/mmol), [^{3}H]cAMP (1.2 TBq/mmol), [^{3}H]DHEA sulfate (520 GBq/mmol), [^{3}H]estradiol-glucuronide (1.6 TBq/mmol), [^{3}H]estrone sulfate (2.0 TBq/mmol), [^{3}H]PGE_2 (7.4 TBq/mmol), [^{14}C]salicylate (2.0 GBq/mmol), [^{14}C]succinate (2.18 GBq/mmol), [^{3}H]taurocholate (111 GBq/mmol), and [^{14}C]TEA (0.13 GBq/mmol) were from PerkinElmer Life Science Products (Boston, MA); [^{3}H]glucuronic acid (185 GBq/mmol), [^{14}C]glutarate (2.0 GBq/mmol), [^{3}H]ibuprofen (18.5 TBq/mmol), [^{14}C]uric acid (1.85 GBq/mmol), and [^{14}C]valproic acid (2.1 GBq/mmol) were from American Radiolabeled Chemicals, Inc. (St. Louis, MO); [^{3}H]acyclovir (333 GBq/mmol), [^{3}H]methotrexate (555 GBq/mmol), and [^{3}H]ochratoxin A (547.6 GBq/mmol) were from Moravek Biochemical Inc. (Brea, CA); [^{3}H]cimetidine (673 GBq/mmol) was from Amersham Pharmacia Biotech (Uppsala, Sweden); DHEA sulfate, estrone sulfate, *N*-methyl-D-glucosamine, *p*-aminohippuric acid, sulfobromophthalein, ibuprofen, furosemide, bumetanide and azidothymidine were from Sigma (St. Louis, MO). All other chemicals and reagents used were of analytical grade and obtained from commercial sources.

Reverse Transcription-PCR and Isolation of hOAT3. EST (expressed sequence tag) database were searched for "query rOAT3", and an EST clone (H20345) was identified. Primers were designed based on the nucleotide sequence of H20345: forward primer, 5′-AAGTTCATCACCATCCTCTC-3′; reverse primer, 5′-GATCCCGTAAGATGATATTG-3′. Using this set of primers, we performed reverse transcription-PCR using the human kidney poly(A)$^+$ RNA. The protocol for PCR was as follows: 94°C for 10 s, 57°C for 30 s, 72°C for 30 s, 35 cycles. The ^{32}P-labeled dCTP probe was synthesized from the PCR clone and used for the screening of a human kidney cDNA library. A nondirectional cDNA library was prepared from human kidney poly(A)$^+$ RNA (CLONTECH, Palo Alto, CA) using the Superscript Choice system (Life Technologies, Gaithersburg, MD), and the cDNAs were ligated into λZipLox *Eco*RI arms. Replicated filters of a phage library were hybridized overnight at 37°C in a hybridization solution [50% formamide, 5× standard saline citrate (SSC), 3× Denhardt's solution, 0.2% SDS, 10% dextran sulfate, 0.2 mg/ml denatured salmon sperm DNA, 2.5 mM sodium pyrophosphate, 25 mM MES, and 0.01% Antifoam B, pH 6.5], and washed at 37°C in 0.1× SSC and 0.1% SDS.

Sequence Determination. Specially synthesized oligonucleotide primers were used for the sequencing of the hOAT3 cDNA by the dye-termination method using ABI Prism 310.

cRNA Synthesis and Uptake Experiments using *Xenopus laevis* Oocytes. cRNA synthesis and uptake measurements were performed as described previously (Kusuhara et al., 1999). The capped cRNA was synthesized in vitro using T7 RNA polymerase from the plasmid DNA linearized with *Xba*I. Defolliculated oocytes were injected with 10 ng of the capped hOAT3 cRNA and incubated in Barth's solution (88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.4 mM $CaCl_2$, 0.8 mM $MgSO_4$, 2.4 mM $NaHCO_3$, and 10 mM HEPES) containing 50 μg/ml gentamicin at 18°C. After 2 to 3 days of incubation, uptake and efflux experiments were performed at room temperature in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES, pH 7.4) as described elsewhere (Kusuhara et al., 1999). We repeated each experiment more than two times to confirm the results. The representative results are shown in Figs. 3 to 5. The kinetic parameters were obtained by an iterative nonlinear least-squares method using a MULTI program (Yamaoka et al., 1981).

Northern Blot Analysis. A commercially available hybridization blot containing poly (A)$^+$ RNA from various human tissues (human 12-lane multiple tissue Northern blot; CLONTECH) was used for the Northern blot analysis for hOAT3. We used full-length of hOAT3 cDNA as a probe. The master blot filter was hybridized with the probe 1 h at 68°C according to the manufacture's instructions. The filter was washed finally in a high stringency condition (0.1× SSC and 0.1% SDS at 68°C).

Immunohistochemical Analysis. For immunohistochemical analysis, rabbits were immunized with a keyhole limpet hemocyanin-conjugated synthesized peptide, CRIPLQPHGPGLGSS, corresponding to cysteine and the 14 amino acids of the COOH terminus of hOAT3. Two-micrometer wax sections of nephrectomized human kidney were processed for light microscopic immunohistochemical analysis, using the streptavidin-biotin-horseradish peroxidase complex technique (LSAB kit; DAKO, Carpinteria, CA). The renal tissue was from a tumor patient and approved by the Kyorin University Institutional Review Board to be used for medical study. Sections were dewaxed, rehydrated, and incubated with 3% H_2O_2 for 10 min to eliminate endogenous peroxidase activity. After rinsing in 0.05 M Tris-buffered saline containing 0.1% Tween-20, sections were treated with 10 μg/ml of primary rabbit polyclonal antibodies (4°C overnight). Thereafter, the sections were incubated with the secondary antibody, biotinylated goat polyclonal antibody against rabbit immunoglobulin (DAKO), diluted 1:400 for 30 min with horseradish peroxidase-labeled streptavidin. This step was followed by incubation with diaminobenzidine and hydrogen peroxide. The sections were counterstained with hematoxylin and examined by light microscopy. For preabsorption experiment, the hOAT3 peptide (200 μg/ml) was added to the hOAT3-specific antibody solution and incubated overnight at 4°C. Using this preabsorbed antibody, the immunohistochemistry was performed as described above.

Fluorescent in Situ Hybridization Analysis. Lymphocytes isolated from human blood were cultured in a-minimal essential medium (a-MEM) supplemented with 10% fetal calf serum and phytohemagglutinin at 37°C for 68 to 72 h. The lymphocyte cultures were treated with 0.18 mg/ml bromodeoxyuridine (Sigma) to synchronize the cell population. The synchronized cells were washed three times with serum-free medium to release the block and recultured at 37°C for 6 h in a-MEM with 2.5 mg/ml thymidine (Sigma). Cells were harvested and slides were made using standard procedures including hypotonic treatment, fixation, and air-drying. The hOAT3 probe was biotinylated with dATP using the BRL BioNick labeling kit (15°C, 1 h) (Heng et al., 1992). The slides were then baked at 55°C for 1 h. After the RNase treatment, the slides were denatured in 70% formamide in 2× SSC for 2 min at 70°C followed by dehydration with ethanol. Proves were denatured at 75°C for 5 min in a hybridization mixture consisting of 50% formamide and 10% dextran sulfate. Probes were applied to the denatured chromosomal slides. After overnight hybridization, the slides were washed and screened, as well as amplified. The fluorescent in situ hybridization

(FISH) signals and 4,6-diamidino-2-phenylindole (DAPI) banding patterns were recorded separately by taking photographs, and the assignment of the FISH mapping data to chromosomal bands was achieved by superimposing FISH signals with DAPI-banded chromosomes (Heng and Tsui, 1993)

Statistical Analysis. Data are expressed as mean ± S.E.M. Statistical differences were determined using Student's t test. Differences were considered significant at the level of $p < 0.05$.

Results

EST database search identified an EST clone, H20345, that showed significant identity to rOAT3. Using H20345 as a probe, one positive clone (hOAT3) was isolated by screening 240,000 plaques from the human kidney cDNA library. Although the hOAT3 has been reported (Race et al., 1999), it has no transport function. To compare with our hOAT3 gene, we employed hOAT3* that was cloned by Race et al. hOAT3 cDNA consisted of 2179 base pairs encoding a 543-amino-acid residue protein. Figure 1 shows the deduced amino acid sequence of hOAT3 in the alignment with those of hOAT3*, hOAT1, rOAT2, and hOAT4. The amino acid sequence of hOAT3 showed 85, 51, 36, and 44% identity with those of hOAT3*, hOAT1, rOAT2, and hOAT4, respectively. hOAT3 also showed significant identity with human OCT1 (39%) and OCT2 (37%) (Gorboulev et al., 1997) and rat OCT3 (35%) (Kekuda et al., 1998). The SOSUI analysis (Kyte and Doolittle, 1982) predicted 12 membrane-spanning domains in hOAT3 (hydropathy plot not shown). As in the case of members of OAT and OCT families, *N*-glycosylation sites (residues 54, 81, 86, and 102) and protein kinase C-dependent phosphorylation sites (residues 266, 528, and 511) are suggested in the sequence of hOAT3 (Fig. 1).

The expression of hOAT3 mRNA in human tissues was investigated (Fig. 2). A strong mRNA band was detected in the kidney (2.2 kilobase pairs) and weak bands were also detected in the skeletal muscle and brain. No hybridization signals were detected with mRNAs isolated from other tissues, including the heart, thymus, spleen, liver, small intestine, lung, and peripheral blood leukocytes.

Using the *Xenopus laevis* oocyte expression system, we investigated the transport characteristics using various substances. Figure 3 shows the transport properties of estrone

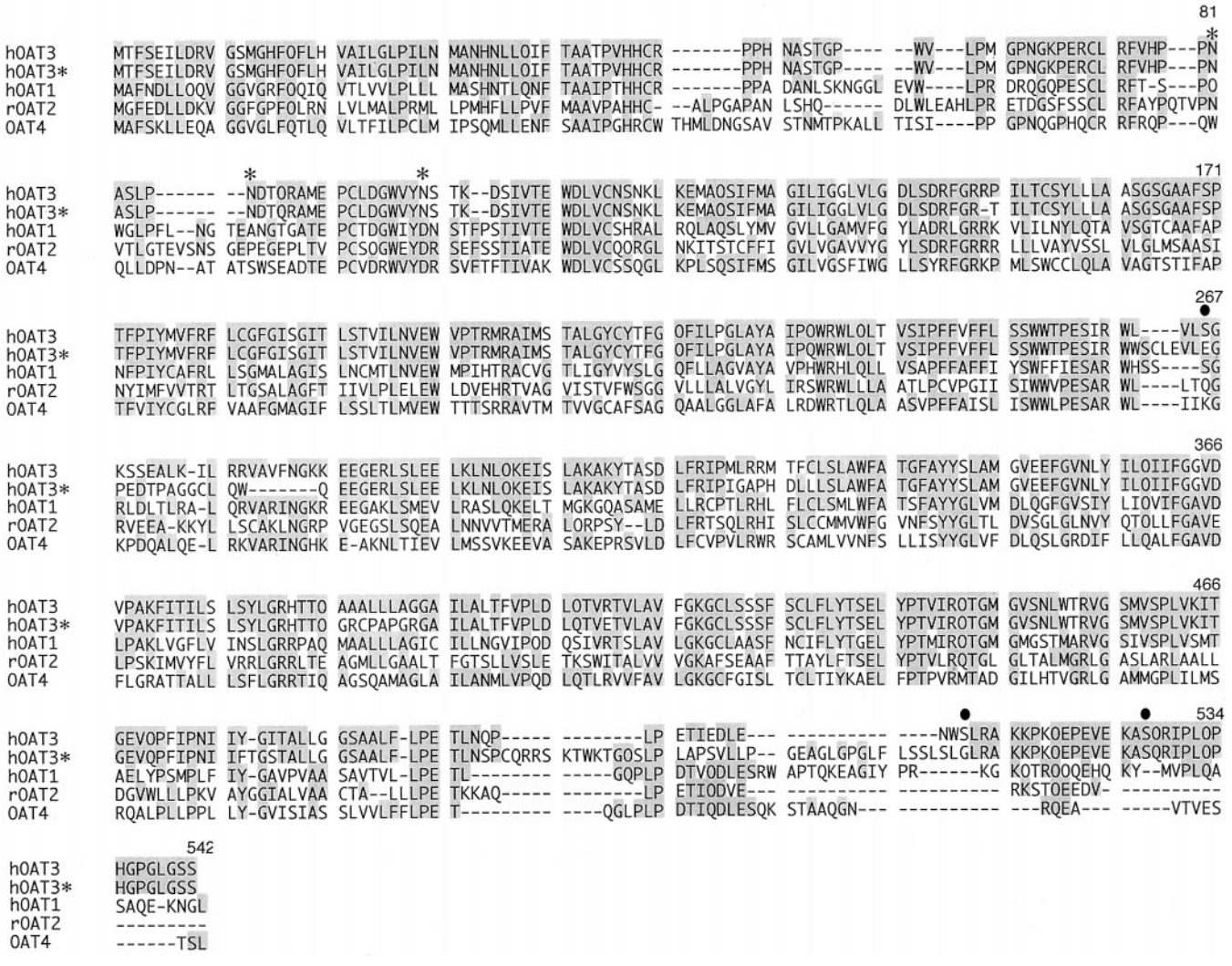

Fig. 1. The amino acid sequence of hOAT3 aligned with those of hOAT3, hOAT1, rOAT2, and hOAT4. Boxed residues indicate conserved residues in at least two transporters. *, putative *N*-linked glycosylation sites in OAT3; ●, putative protein kinase C phosphorylation sites. The probe (H20345) that we used for the screening of OAT3 corresponds to the amino acid residues 370 to 480 of hOAT3. The same region was used for the Northern blot analysis shown in Fig. 2.

sulfate via hOAT3. The cell-associated count of [^{3}H]estrone sulfate increased linearly until 3 h of incubation in hOAT3-expressing oocytes. This result indicates that hOAT3 not only binds but also translocates estrone sulfate into the cytoplasm (Fig. 3A). The uptake rate of estrone sulfate via hOAT3 was not affected by the replacement of the extracellular sodium with lithium, choline, or *N*-methyl-D-glucosamine (Fig. 3B). In the experiment shown in Fig. 3C, the *trans*-stimulatory effect of estrone sulfate on OAT3-mediated efflux of estrone sulfate was examined. The efflux of estrone sulfate was not *trans*-stimulated in the presence of extracellular estrone sulfate (0.5, 5, and 50 μM).

The concentration dependence of hOAT3-mediated uptake of [^{3}H]estrone sulfate, [^{14}C] PAH, [^{3}H]methotrexate, and [^{3}H]cimetidine was examined (Fig. 4). hOAT3-mediated uptake of these four compounds showed saturable kinetics and followed the Michael-Menten equation. Nonlinear regression analyses yielded K_m values of 3.1 ± 0.8 μM, 87.2 ± 11.1 μM, 10.9 ± 1.7 μM, and 57.4 ± 10.9 μM and V_{max} values of 5.7 ± 1.0 pmol/h/oocytes, 20.4 ± 3.6 pmol/h/oocytes, 2.8 ± 0.3 pmol/h/oocytes, and 92.2 ± 11.2 pmol/h/oocytes for estrone sulfate, PAH, methotrexate, and cimetidine, respectively.

The influx of various organic tracers via hOAT3 was investigated (Table 1). The uptake rates of [^{3}H]estrone sulfate, [^{3}H]DHEA sulfate, [^{3}H]ochratoxin A, [^{14}C]*p*-aminohippurate, [^{3}H]methotrexate, [^{3}H]cimetidine, [^{3}H]prostaglandin E_2, [^{3}H]estradiol glucuronide, [^{3}H]taurocholate, [^{14}C] glutarate, [^{14}C] salicylate, [^{3}H]cAMP, and [^{14}C]uric acid in oocytes expressing hOAT3 were much higher than those of control oocytes. No significant uptake of [^{14}C]valproic acid, [^{14}C]succinate, [^{3}H]glucuronic acid, [^{3}H]ibuprofen, [^{14}C]TEA, and [^{3}H]acyclovir was detected.

To investigate substrate selectivity of hOAT3, an inhibition study was performed. The *cis*-inhibitory effect of various compounds (5 μM) on hOAT3-mediated [^{3}H]estrone sulfate (50 nM) uptake was investigated (Fig. 5A). Five micromolar unlabeled probenecid, DHEA-s, indomethacin, ibuprofen, diclofenac, furosemide, bumetanide, and cholate exhibited def-

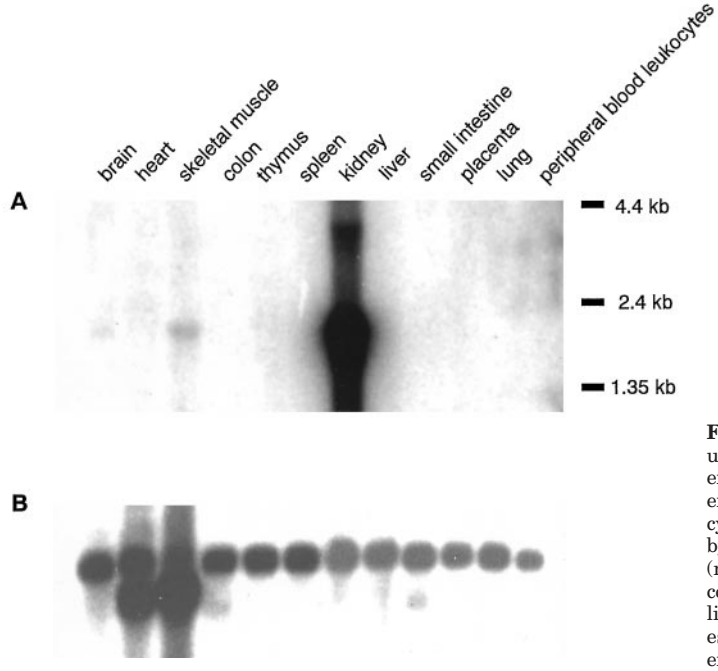

Fig. 2. Northern blot analysis of hOAT3 (A) and β-actin (B). Human multiple tissue blot (CLONTECH) that contains 2 μg of poly(A)$^+$ RNA from 12 human tissues in each of the lanes was probed with a ^{32}P-labeled hOAT3 cDNA fragment and was washed in a high stringent condition.

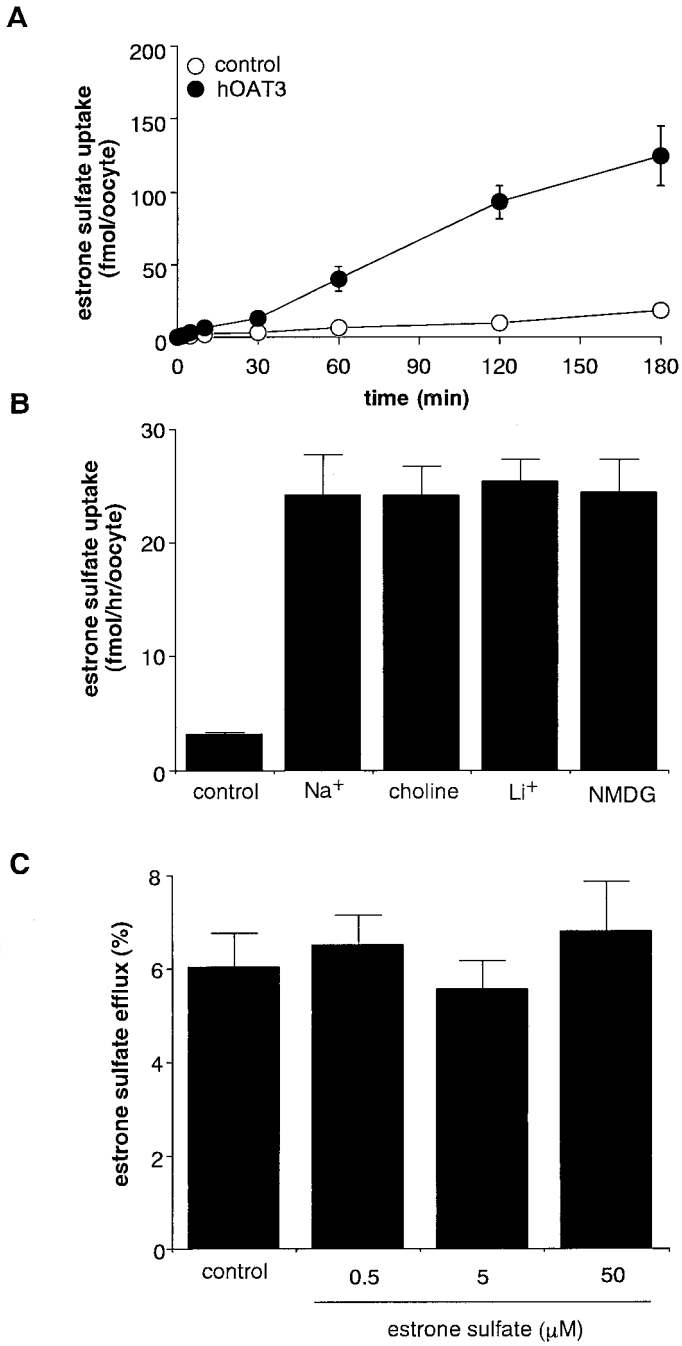

Fig. 3. The transport properties of estrone sulfate via hOAT3. A, the uptake of 50 nM [^{3}H]estrone sulfate in control oocytes (○) and OAT3-expressing oocytes (●) was measured during 3 h of incubation. B, effect of extracellular cation on [^{3}H]estrone sulfate uptake in *Xenopus laevis* oocytes expressing hOAT3. The uptake rate of [^{3}H]estrone sulfate (50 nM) by control oocytes or hOAT3-expressing oocytes for 1 h was measured (mean ± SEM) in the presence or absence of extracellular Na^+. Extracellular Na^+ was replaced with equimolar concentration of lithium, choline, or *N*-methyl-D-glucamine. C, the lack of *trans*-stimulatory effect of estrone sulfate on hOAT3-mediated efflux of estrone sulfate. Oocytes expressed with hOAT3 was incubated with 100 nM [^{3}H]estrone sulfate for 90 min and transferred to the ND96 solution (control) or ND96 containing 0.5, 5, or 50 μM unlabeled estrone sulfate. The effluxed amount of estrone sulfate during 90 min was shown as a percentage of the preloaded amount (mean ± SEM).

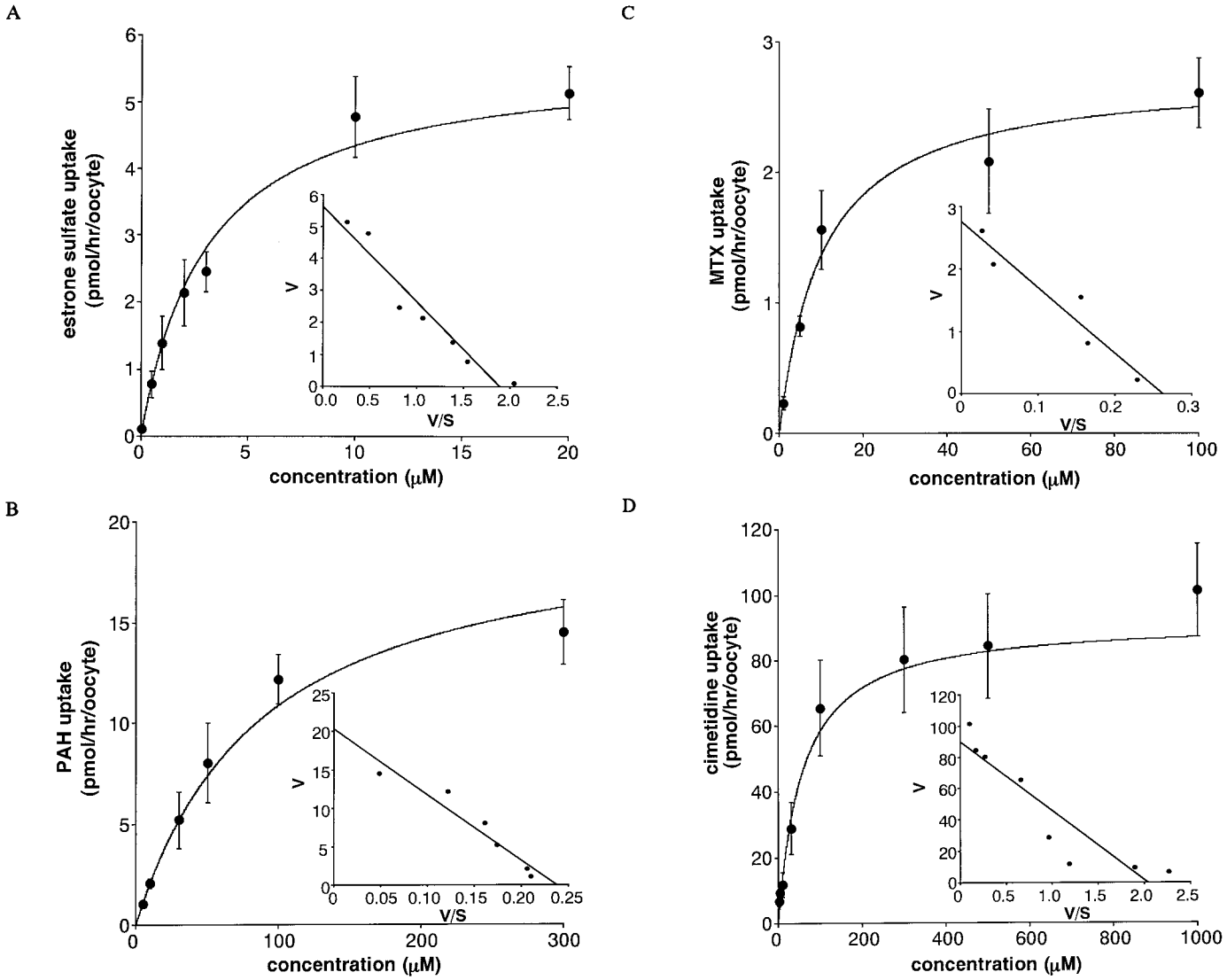

Fig. 4. Concentration dependence of hOAT3-mediated uptake of [^{3}H]estrone sulfate (A), [^{14}C]*p*-aminohippurate (B), [^{3}H]methotrexate (C), and [^{3}H]cimetidine (D). The uptake rates of four compounds by control or hOAT3-expressing oocytes for 1 h were measured at variable concentrations (mean ± SEM; n = 8–10). hOAT3-mediated transport was determined by subtracting the transport velocity in control oocytes from that in hOAT3-expressing oocytes.

inite inhibitory potency. Salicylate, glutarate, penicillin G, sulfobromophthalein, taurocholate, corticosterone (a neutral steroid hormone), quinidine, and TEA bromide exhibited modest inhibitory activity. In contrast, ouabain and guanidine did not show inhibitory activity. The IC_{50} values of estrone sulfate, MTX, PAH, and cimetidine were about 3.0×10^{-6} M, 4.2×10^{-5} M, 1.8×10^{-4} M, and 7.0×10^{-5} M, respectively (Fig. 5B).

Light microscopy of 2-μm wax sections demonstrated that there was specific immunostaining of hOAT3 in the proximal tubular cells (Fig. 6A). There was no staining of hOAT3 in Bowman's capsule, glomerular cells, distal tubules, or cortical collecting duct. Under high magnification, hOAT3 was located in the basolateral membranes of the proximal tubules (Fig. 6C). hOAT3 immunoreactivity was not observed in the medulla (Fig. 6B). By preincubation of the antibody with hOAT3 peptide, the immunoreactivity was completely diminished (Fig. 6D). The specificity of the antibody for hOAT3 was verified by these results.

Under the conditions used, FISH detection efficiency was approximately 47% using this probe (among the 100 checked mitotic figures, 47 showed hybridization signals on one pair of chromosomes). When DAPI banding was used to identify the specific chromosome, the assignment between signals from the probe and the long arm of chromosome 11 (Fig. 7A) was obtained. The detailed position was further determined based on 10 photos (Fig. 7B). Therefore, this probe was mapped to the human chromosome 11, region q12–q13.3.

Discussion

In the present study, we report on the isolation and characterization of multispecific hOAT3. hOAT3 encodes a 543-amino-acid residue protein, that shows 36 to 51% identity to hOAT1, rOAT2, hOCT1, hOCT2, and rOCT3.

In the previous studies using membrane vesicles, kidney slices, and in situ microperfusion, most of the basolateral uptake of hydrophobic organic anions have been attributed to

TABLE 1

Uptake of various ^{3}H- or ^{14}C-labeled compounds by hOAT3-expressing oocytes

The uptake experiments were performed at the concentration indicated for 1 h. Values are mean ± SE of 8~12-oocyte determinations. The significance between water-injected and hOAT3-cRNA-injected oocytes was determined by unpaired *t* test. The chemical concentrations used were [a] 50 nM, [b] 100 nM and [c] 10 μM. The units of measure were femtomoles per oocyte per hour for ^{3}H tracers and picomoles per oocyte per hour for ^{14}C tracers.

Tracer	Chemical Concentration Used	Control	hOAT3	*p* Value
[^{3}H]DHEA sulfate	50 nM	1.7 ± 0.1	33.7 ± 11.1	<0.01
[^{3}H]Estrone sulfate	50 nM~20 μM	4.5 ± 0.2	23.9 ± 3.0[a]	<0.01
[^{3}H]Estradiol glucuronide	50 nM	8.2 ± 0.5	36.2 ± 6.1	<0.01
[^{14}C]Glutarate	10 μM	2.9 ± 0.2	5.4 ± 1.4	<0.01
[^{3}H]PGE_2	1 μM	0.2 ± 0.1	0.8 ± 0.1	<0.01
[^{3}H]Taurocholate	100 nM	94.8 ± 10.7	275.6 ± 37.7[b]	<0.01
[^{3}H]Methotrexate	50 nM~100 μM	7.9 ± 1.7	17.7 ± 3.4[b]	<0.01
[^{3}H]Cimetidine	50 nM~100 μM	7.1 ± 0.9	82.8 ± 6.0	<0.01
[^{14}C]*p*-Aminohippurate	10 μM~300 μM	0.5 ± 0.1	9.2 ± 1.3[c]	<0.01
[^{3}H]Ochratoxin A	50 nM	8.8 ± 0.8	30.8 ± 3.0	<0.01
[^{3}H]cAMP	100 nM	98.9 ± 5.9	140.1 ± 7.7	<0.05
[^{14}C]Salicylate	100 nM	15.7 ± 0.4	19.5 ± 1.0	<0.05
[^{14}C]Uric acid	10 μM	0.6 ± 0.2	1.4 ± 0.4	<0.05
[^{14}C]Valproic acid	5 μM	5.5 ± 0.2	5.1 ± 0.5	N.S.
[^{14}C]Succinate	10 μM	5.1 ± 0.5	6.4 ± 0.2	N.S.
[^{3}H]Glucuronate	10 nM	5.1 ± 0.7	4.7 ± 0.4	N.S.
[^{3}H]Ibuprofen	10 nM	21.4 ± 2.1	19.5 ± 1.0	N.S.
[^{3}H]Acyclovir	1 μM	0.2 ± 0.0	0.2 ± 0.0	N.S.
[^{14}C]TEA	30 μM	2.2 ± 0.5	3.0 ± 0.6	N.S.

N.S., not significant.

the function of the PAH transporter. In 1997, the PAH/decarboxylate exchanger, rOAT1, was cloned (Sekine et al., 1997; Sweet et al., 1997), and subsequently three isoforms (rOAT2, rOAT3, and hOAT4) were identified (Sekine et al., 1998; Kusuhara et al., 1999; Cha et al., 2000). In addition to the OAT family, two other multispecific organic anion transporter families were identified, namely the Mrp (multidrug resistance associated protein) family (Buchler et al., 1996; Ito et al., 1997) and the oatp (organic anion transporting polypeptide) family (Jacquemin et al., 1994; Noe et al., 1997). Despite the rapid progress in molecular biology of organic anion transporters, the physiological role of each molecule in renal organic anion transport remains to be elucidated. Among these multispecific organic anion transporters, only rOAT1 (Nakajima et al., 2000), rOAT3 (R. Kojime, T. Sekine, M. Kauachi, S. H. Cha, Y. Suzuki, and H. Endou, submitted)

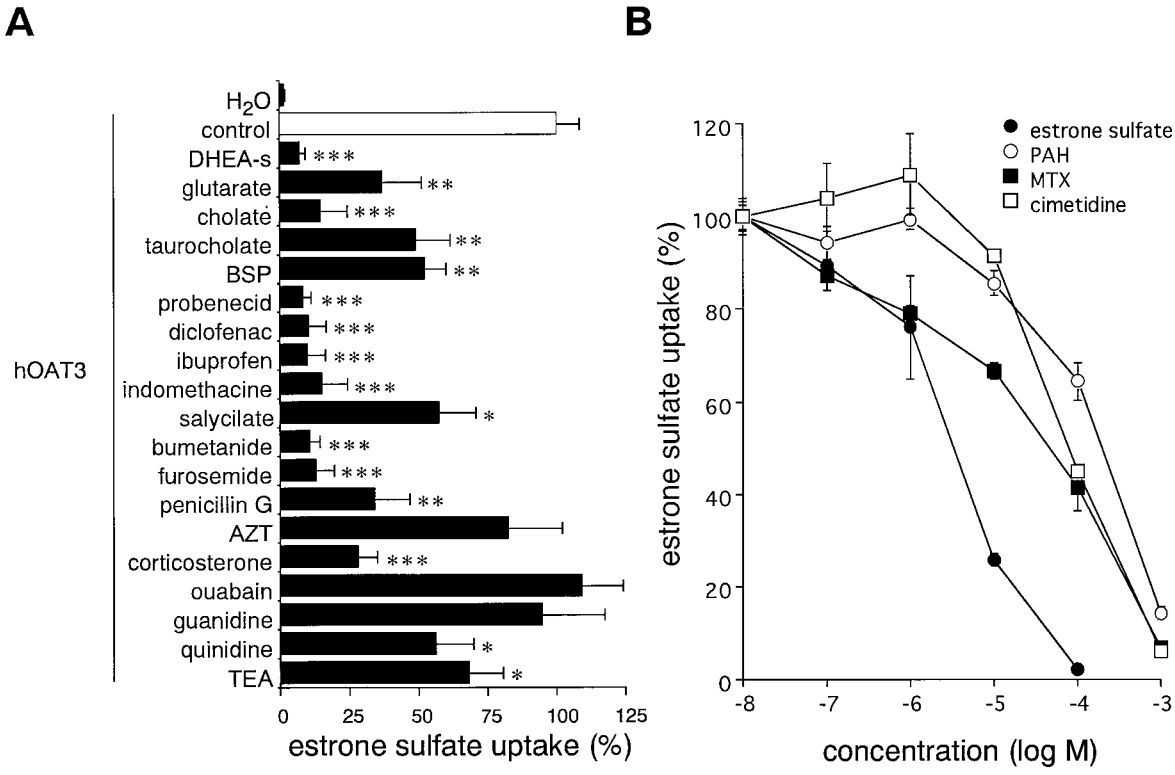

Fig. 5. Inhibition of hOAT3-mediated [^{3}H]estrone sulfate uptake by various compounds (A) and inhibition profile by estrone sulfate, MTX, PAH, and cimetidine (B). The uptake rate of [^{3}H]estrone sulfate (50 nM) by hOAT3-expressing oocytes or noninjected oocytes was determined in the absence or presence of 5 μM inhibitors. DHEA-s, DHEA sulfate; BSP, sulfobromophthalein; AZT, azidothymidine (zidovudine). The values were expressed as a percentage of [^{3}H]estrone sulfate uptake in hOAT3-expressing oocytes in the absence of the inhibitor (mean ± SEM; $n = 8–10$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

and Mrp1 (Raggers et al., 1999) are localized in basolateral membranes of proximal tubular cells. Mrp1 mediates the extrusion of organic anions from cells, and it does not function for the cellular uptake of organic anions. OAT1 and OAT3 are now the only multispecific organic anion transporters responsible for the basolateral uptake of organic anions from the peritubular plasma.

For a comprehensive understanding, determination of the contribution of each transporter in the renal organic anion transport system is required. OAT1 has been considered the predominant organic anion transporter in the basolateral membrane (Hosoyamada et al., 1999); however, the role of OAT1 might be overestimated, particularly in the human kidney. First, it should be noted that most of the characterization of renal organic anion transporters have been obtained from the experiments in which PAH was used as a tracer, and substrate selectivity was examined in terms of inhibitory effects of test substances. The results of such experiments primarily reflect the functional characteristics of an organic anion transporter, OAT1, which mediates the high-capacity transport of PAH. Second, before the identification of OAT isoforms, transport characteristics of OAT3 might have not been determined. Third, there seems to exist differences in species among OATs. hOAT1 is also a PAH/decarboxylate exchanger and mediates the high-affinity transport of PAH; however, it was suggested that the ability of hOAT1 to translocate organic anions might be limited. Lu et al. (1999) reported that hOAT1 does not translocate methotrexate or PGE_2, both of which are transportable substrates of rOAT1. They suggested that hOAT1 shows narrow substrate selectivity for organic anions. A similar observation of difference in species was reported in the oatp family, another multispecific organic anion transporter family, in which the difference in species is more apparent. In the rat liver, oatp1 and/or oatp2 are predominant isoforms; in contrast, the corresponding human homolog, OATP, is weakly expressed in the human liver, where a distinct isoform LST1 (liver-specific transporter 1), seems to be the predominant isoform (Abe et al., 1999). Multispecific organic anion transporters, which function primarily in the detoxification and elimination of various exogenous compounds and metabolites, may evolve differently depending on the circumstance. Although immunoreactivity of OAT1 (S2) and OAT3 (S1 > S2 = S3) are observed in basolateral membranes of proximal tubules, OAT1 and OAT3 show different K_m values for the same substrates. The K_m values of PAH for each of these trans-

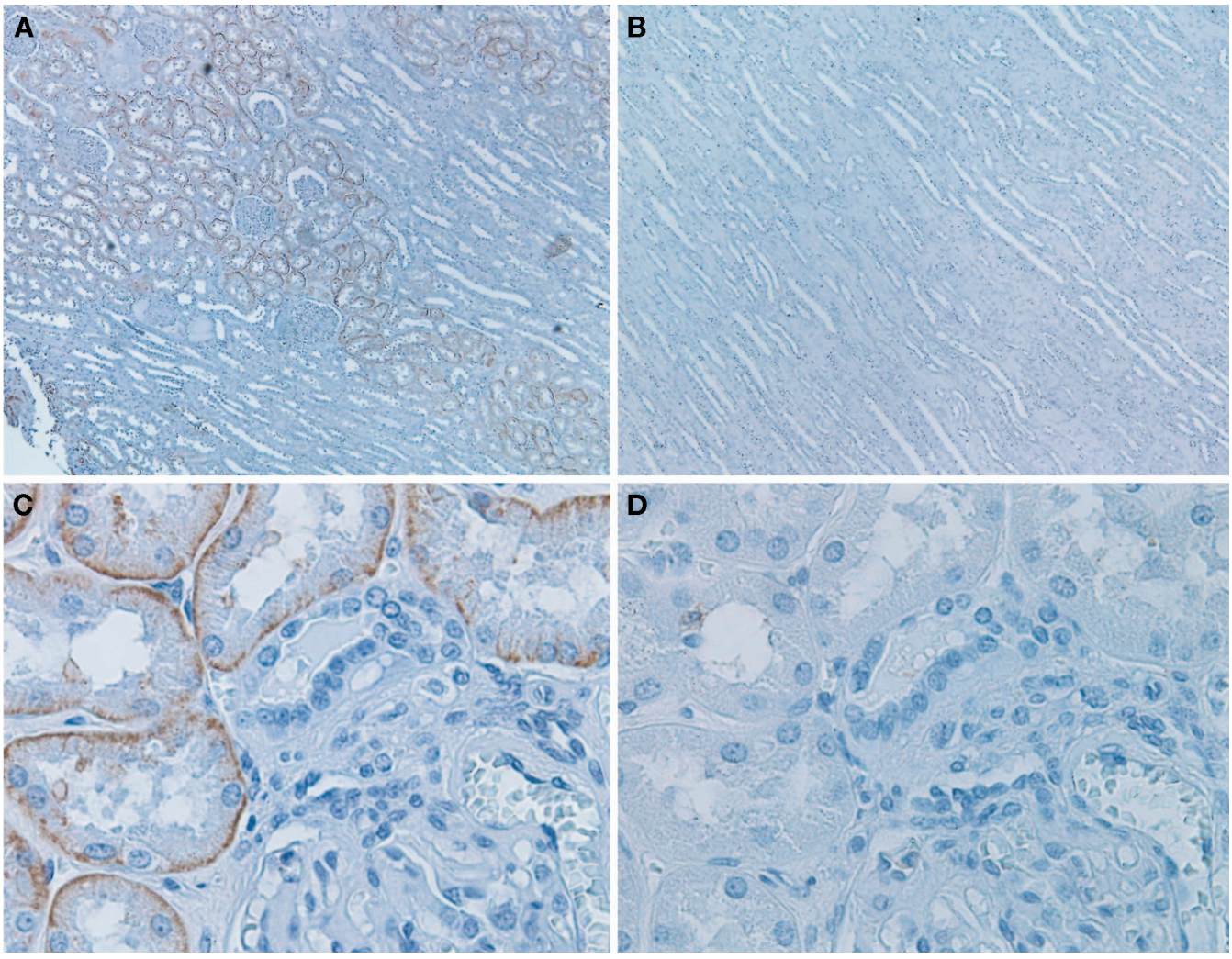

Fig. 6. Immunohistochemical analysis of hOAT3 in the kidney of human. Two-micrometer sections were incubated with polyclonal antibody against hOAT3. Basolateral membrane of proximal tubule was stained (A and C) and no staining was observed in medulla (B). Immunoreactivity was completely abolished by pretreatment of antibody with hOAT3 oligopeptide (D). (A and B, 40×; C and D, 400×)

porters were: rOAT1, 14 μM; rOAT3, 65 μM; hOAT1, 9 μM; and hOAT3, 87 μM. It suggests that these transporters would have different contributions in tubular secretion. Concerning the circumstance of renal blood circulation, broad distribution of hOAT3 in renal proximal tubule increases the opportunity to contact circulated organic anionic substrates with hOAT3. The contributions of hOAT1 and hOAT3 in the renal organic anion transport systems should be clarified using human kidney samples and/or in vivo clearance studies with relatively specific inhibitors for hOAT1 and hOAT3.

Recently, Race et al. (1999) have reported on the hOAT3* gene isolated from the human kidney. The homology between rOAT3 and hOAT3* was only 68%. In particular, the C terminus of hOAT3* was longer than that of rOAT3. In addition, they could not show any functional properties of the tested PAH, urate oxalate (organic anionic substrates), and TEA (organic cationic substrate). The deduced amino acid sequence of our hOAT3 showed 85% identity with that of hOAT3*. The major difference in amino acid sequence between hOAT3 and hOAT3* lies in the four regions (residues 263–286, 334–341, 399–406, and 513–527 in hOAT3 residue; see Fig. 1). In particular, hOAT3* possesses 25 more amino acid insertions than hOAT3 in the near portion of the C terminus, which may alter the conformation of the protein and interfere with the binding of the substrate to hOAT3*. It remains to be elucidated whether hOAT3 and hOAT3* are formed by alternative splicing or are encoded by different genes.

hOAT3 mediates the high-affinity transport of methotrexate (MTX) (K_m = 10.9 μM). MTX is an antineoplastic agent used in the treatment of acute lymphoblastic leukemia (Balis et al., 1998) and choriocarcinomas (Ohno et al., 1993). MTX is

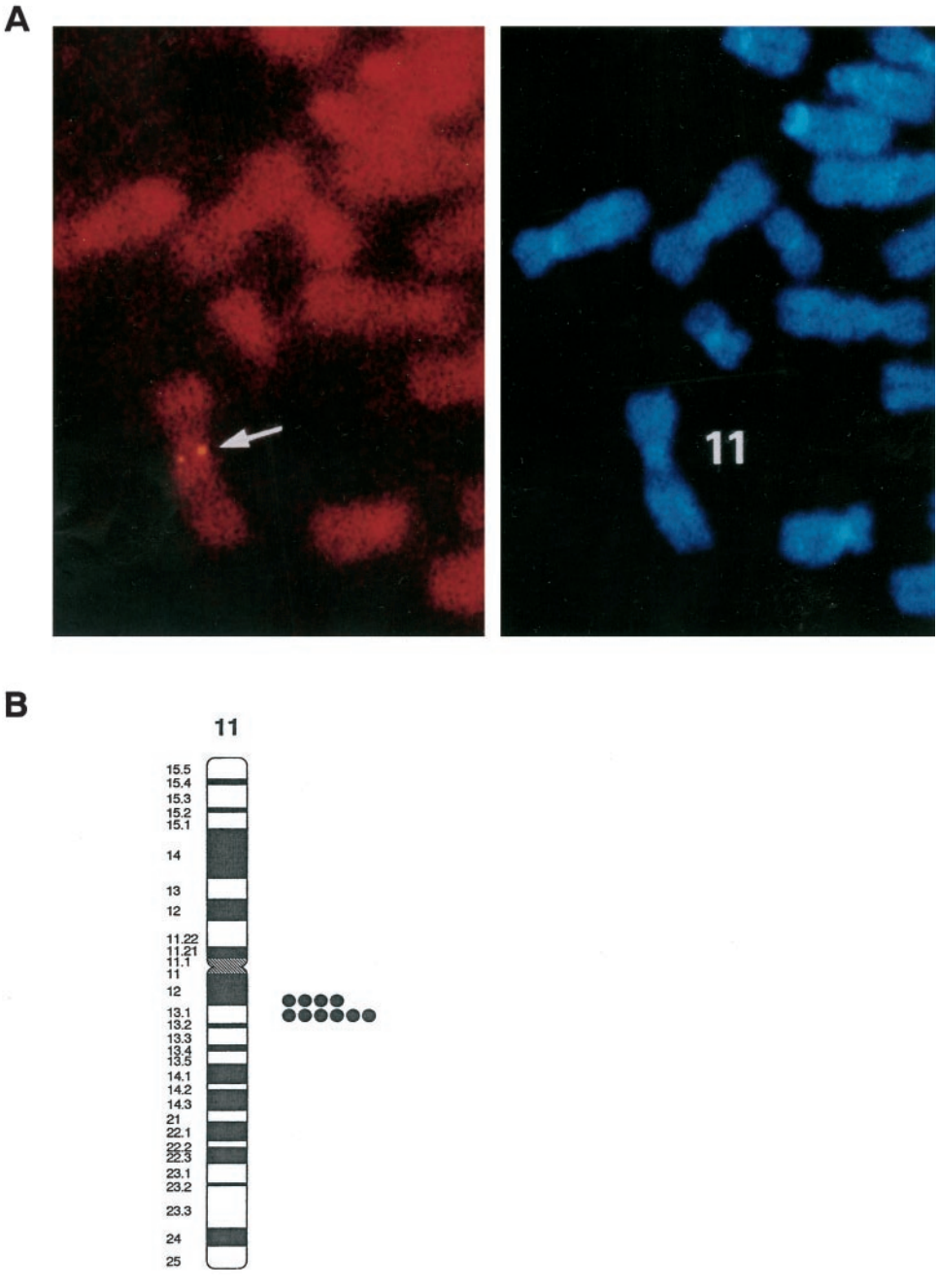

Fig. 7. Diagram of FISH mapping result for hOAT3. A, example of FISH mapping of probe hOAT3. Left, the FISH signals on human chromosome; right, the same mitotic figure stained with 4,6-diamino-2-phenylindole to identify human chromosome 11. B, each dot represents double FISH signals detected on human chromosome 11.

also used in the treatment of nonneoplastic diseases, such as psoriasis (Zonneveld et al., 1996), rheumatoid arthritis (Zonneveld et al., 1996), systemic lupus erythematosus (Ravelli et al., 1998) and dermatomyositis (Itoh et al., 1999). Because MTX manifests potentially toxic effects, such as suppression of bone marrow (Iqbal and Ali, 1993) and intestinal epithelial damage (Nakamaru et al., 1998) under high plasma concentration, understanding of the pharmacokinetics of MTX is required. In humans, MTX is mainly excreted in the urine in the unchanged form via both glomerular filtration and tubular secretion. It has been reported that concomitant use of MTX with acidic drugs, such as nonsteroidal anti-inflammatory drugs and β-lactam antibiotics, causes severe suppression of bone marrow. This seems to be the result of competitive inhibition of the process of the renal organic anion transport system. Because hOAT3 seems to be the major route for the basolateral uptake of MTX, information on the substrate specificity of hOAT3 is important for the use of MTX, particularly for the treatment of patients with decreased renal function.

hOAT3 shows overlapping substrate selectivity with hOAT1. Phylogenetic analysis reveals that hOAT3 is located nearest to hOAT1, which may underlie the similar substrate selectivity. There are, however, distinct differences in substrate recognition between hOAT1 and hOAT3. Oocytes expressing hOAT3 mediates the transport of estrone sulfate, estradiol-glucuronide. On the other hand, oocytes expressing hOAT1 showed little or no transport of PGE_2, methotrexate, and taurocholate (data not shown). hOAT3 can mediate the transport of organic anions with bulky side groups, compared with hOAT1. Interestingly, hOAT3 mediates the high-affinity transport of cimetidine, a cationic substance. It is well known that cimetidine is a bisubstrate type of organic anion and cation transporter. Ullrich et al. (1993) studied the recognition of bisubstrates using a renal organic anion transporter employing the stop-flow peritubular-capillary microperfusion method. They showed the importance of hydrophobicity and partial charge, but not that of net charges of the substrates. In addition, TEA (5 μM) and quinidine showed significant inhibitory effects on the hOAT3-mediated transport of estrone sulfate. Thus, the charge recognition of hOAT3 seems to be not so stringent. Oatp1, another isoform of the other multispecific organic anion transporter family, has been also shown to mediate the transport of an organic cation, and Eckhardt et al. (1999) proposed that oatp1 be called a polyspecific transporter. Thus, the substrate selectivity of hOAT3 is also similar to those of oatp families.

The present study indicates that hOAT3 mRNA is exclusively expressed in the kidney and weak bands could be seen in the skeletal muscle and brain. hOAT3 mRNA expression in skeletal muscle is quite different from rOAT3 mRNA expression. At present, it is not clear whether hOAT3 functions in nonsecreting tissue, such as skeletal muscle.

FISH analyses revealed that the hOAT3 gene is located at the locus of the human chromosome 11q12–q13.3. This chromosomal localization is slightly different from that of hOAT3*, which was reported to be located in chromosome 11q11.7.

In conclusion, we report on the identification and characterization of human OAT3. In the human kidney, hOAT3 seems to play important roles in the basolateral uptake of organic anions in proximal tubular cells and to be a key molecule determining the pharmacokinetics of anionic drugs in human.

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