We have cloned a cDNA coding for a novel member of organic anion transporter, designated OAT-K1, expressed specifically in the kidney of rats. The rat OAT-K1 cDNA (2788 base pairs) had an open reading frame encoding for a 669-amino acid protein (calculated molecular mass of 74 kDa) which shows 72% identity with the cloned rat liver organic anion transporter, oatp. Northern hybridization and reverse transcription-coupled polymerase chain reaction revealed that the rat OAT-K1 messenger RNA transcript is expressed predominantly in the kidney. By use of stable LLC-PK1 cell monolayers transfected with the rat OAT-K1 cDNA, the transporter was suggested to mediate basolateral uptake of methotrexate, an anionic anticancer drug, but not taurocholate, p-aminohippurate, prostaglandin E2, and leukotriene C4. The methotrexate transport by rat OAT-K1 was unaffected by the presence of Na+ or Cl− gradient. The methotrexate accumulation by the OAT-K1-expressing cells showed saturability with the apparent \( K_m \) value of 1.0 \( \mu M \). Folate, sulfobromophthalein, and 4,4′-diansathiocyanostilbene-2,2′-disulfonic acid (DIDS) inhibited the methotrexate accumulation markedly. These findings suggest that the rat OAT-K1 is localized in the basolateral membranes of renal tubules, where it mediates renal clearance of methotrexate from the blood.

Secretion of anionic endogenous substances and xenobiotics is an important function of the liver and kidney. However, these tissues have different physiological and pharmacological roles in the secretion of organic anions. In particular, bile formation is the most important transport process of hepatocytes. Bile acids and other anionic drugs are taken up by the hepatocytes from portal blood via specific transport systems localized in the sinusoidal plasma membranes. The characteristics and mechanisms of organic anion transport have been studied by use of the perfused liver, isolated hepatocytes, and purified sinusoidal membrane vesicles (1, 2). Conjugated bile acids such as taurocholate and glycocholate enter specifically purified sinusoidal membrane vesicles (1, 2). Conjugated bile acids such as taurocholate and glycocholate enter specifically purified sinusoidal membrane vesicles (1, 2). Conjugated bile acids such as taurocholate and glycocholate enter specifically purified sinusoidal membrane vesicles (1, 2). Conjugated bile acids such as taurocholate and glycocholate enter specifically purified sinusoidal membrane vesicles (1, 2).

**EXPERIMENTAL PROCEDURES**

Reverse Transcription-coupled PCR and cDNA Sequencing—Degenerated PCR primers based on the amino acid sequence of rat oatp (4) are as follows: sense strand, 5′-CCGATCTGC(T)/GC(A/C/T)/GG(T/C/T)CT/AG(T)/AC(A/C/T)/AA-3′ (corresponding to amino acid sequence 28–33); antisense strand, 5′-CCGATCCCGCAT(A/G)AA(A/G)AA(A/G)TG(A/G/T)/GG-3′ (corresponding to amino acid sequence 106–111). One \( \mu g \) of poly(A)+ RNA extracted from the kidney cortex of male Wistar rats was reverse-transcribed as reported previously (7). The synthesized cDNA was used for subsequent PCR with a set of degenerate primers (5 µM) according to the following profile: 94°C for 1 min, 50°C for 1 min, 72°C for 2 min, 30 cycles. The PCR products (approximately 270 bp) were cut with EcoRI and BamHI on both ends and ligated into the EcoRI- and BamHI-cut pSPORT1 (Life Technologies, Inc.). Both strands of the subcloned cDNA inserts were sequenced by the chain-termination method with a Sequenase version 2.0 DNA sequencing kit (U.S. Biochemical Corp.).

Screening of cDNA Library—The oligo(dT)-primed directional rat

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) D79981.

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The abbreviations used are: oatp, organic anion transporting polypeptide; PCR, polymerase chain reaction; kb, kilobase; bp, base pair(s); DIDS, 4,4′-diansathiocyanostilbene-2,2′-disulfonic acid; PGT, prostaglandin transporter.
Cloning of a Novel Methotrexate Transporter in the Kidney

Methotrexate (MTX), 4-aminoantipyrine, and 2-deoxy-D-glucose (2-DG) are transported by organic anion transporters (OATs) in various tissues (4, 6). In the present study, we report the cloning of a cDNA encoding a novel MTX transporter expressed primarily in the renal cortex of rat. The close relationship of this rat transporter to human OAT1 and OAT3 was also demonstrated.

**Materials and Methods**

**Northern Blot and PCR Analyses**

For Northern blot analysis, 2.5 μg of poly(A)+ RNA from rat tissues was reverse transcribed and amplified with either a set of specific primers for the nucleotide sequence of rat oatp (sense strand, 5′-GAAGCTGGAAACAATCGAGA-3′ (bases 2134–2153)) or specific primers for the rat OAT-K1 (sense strand, 5′-GAGGACCATAAGGATGTC-3′ (bases 2134–2153)). For reverse transcription-PCR analysis, 1 μg of poly(A)+ RNA from tissues was reverse-transcribed and amplified with either a set of specific primers for the nucleotide sequence of rat oatp (sense strand, 5′-GAAGCTGGAAACAATCGAGA-3′ (bases 2134–2153)) or specific primers for the rat OAT-K1 (sense strand, 5′-GAGGACCATAAGGATGTC-3′ (bases 2134–2153)). As a control, the rat OAT-K1 cDNA was reverse-transcribed and amplified with each set of these primers.

**Results**

**Sequencing of several PCR products originating from the rat kidney cortex revealed that the existence of a PCR clone that was homologous (80% nucleotide identity) to the oatp cDNA. A single cDNA clone coding OAT-K1 was isolated from the rat kidney cDNA library, subcloned into a plasmid (pSPORT1), and sequenced. The OAT-K1 cDNA consists of 2788 bp with an open reading frame encoding a 669-amino acid transporter protein (calculated molecular mass of 74 kDa) and with a poly(A)+ tail (Fig. 1).**

**Northern blotting**

We examined the tissue distribution of OAT-K1 mRNA transcripts by Northern blot analysis (Fig. 3A). Under both low and high stringent conditions, the whole OAT-K1 cDNA probe hybridized with mRNA transcripts from the rat liver (3 kb), rat kidney (5 kb), and heart (3 kb). The probes hybridize in the rat kidney cortex revealed that the existence of a PCR clone that was homologous (80% nucleotide identity) to the oatp cDNA. A single cDNA clone coding OAT-K1 was isolated from the rat kidney cDNA library, subcloned into a plasmid (pSPORT1), and sequenced. The OAT-K1 cDNA consists of 2788 bp with an open reading frame encoding a 669-amino acid transporter protein (calculated molecular mass of 74 kDa) and with a poly(A)+ tail (Fig. 1).

**RESULTS**

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**Fig. 2 A** shows the deduced amino acid sequence of rat OAT-K1 and its alignment with the rat oatp. Rat OAT-K1 showed amino acid identity of 72% with the rat oatp and 35% with the rat matrix F/G (recently defined as a prostaglandin transporter, PGT) (15, 16), but a search of available data bases (GenBank, EMBL, and SWISS-PROT, February 1996) revealed no significant homology with any other cloned membrane transporter proteins. Kyte-Doolittle (17) hydrophathy analysis suggested that rat OAT-K1 has 12 putative membrane-spanning α-helices (Fig. 2B), thereby indicating four potential N-linked glycosylation sites in the extracellular loop. There are three putative CAMP-dependent kinase phosphorylation sites (18) at positions 290, 383, and 644. Three putative protein kinase C phosphorylation sites (18) are present at positions 383, 644, and 648.

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To characterize the transport function of OAT-K1, the accumulation of various anionic drugs was measured by use of the stable transfectant LLC-OAT-K1 monolayers grown on membrane filters. Six transfectants, which appeared to express the rat OAT-K1 mRNA, were isolated, and a transfectant showing the highest mRNA expression was used for the subsequent experiments. As shown in Fig. 4, methotrexate accumulation in the LLC-OAT-K1 monolayers from the ba-
solateral side was enhanced markedly compared with that in
the cell monolayers (LLC-pBK) transfected with the expression
vector lacking an insert of the rat OAT-K1 cDNA. Neither
taurocholate, p-aminohippurate, prostaglandin E\textsubscript{2} nor
leukotriene C\textsubscript{4} was accumulated into the LLC-OAT-K1 mono-
layers. The other five transfectants obtained also showed
transport activity of methotrexate (data not shown). In a
separate experiment, cellular accumulation of [\textsuperscript{3}H]folate (26
pmol/mg protein per 15 min, respectively).

Fig. 5 illustrates the time course of methotrexate accumu-
lation into LLC-OAT-K1 monolayers. Furthermore, the effects of
replacement of Cl\textsuperscript{−} with gluconate caused no significant ef-
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liver was increased extensively to 923, 584, and 224,
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**DISCUSSION**

We have isolated and characterized cDNA encoding a novel
organic anion transporter OAT-K1 expressed specifically in the
kidney of rats. The deduced amino acid sequence of OAT-K1 has 72% identity with the rat oatp (4), another Na\textsuperscript{+}-independent
organic anion transporter identified in the liver.

The rat OAT-K1 mediates basolateral uptake of methotrexate
with a relatively high affinity (apparent K\textsubscript{m} value of 1.0 \textmu M).

We have examined the methotrexate transport by LLC-OAT-K1
monolayers by the cis inhibition method. All drugs were used at
a concentration of 100 \textmu M except for methotrexate which was
used at both 10 and 100 \textmu M. As illustrated in Fig. 7, [\textsuperscript{3}H]metho-
trexate accumulation was inhibited markedly in the presence of
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indicated that methotrexate secretion was affected by other anionic drugs such as p-aminohippurate (21), probenecid (22), and penicillin (23), suggesting the contribution of the organic anion transport system. Recent findings with isolated basolateral membrane vesicles from the kidney suggest that p-aminohippurate is transported via an organic anion/dicarboxylate exchange system (6). However, methotrexate has not been demonstrated to be recognized and transported by the "organic anion/dicarboxylate exchange system" in the renal basolateral membranes. In the present studies, both Northern hybridization and PCR analyses revealed that the rat OAT-K1 mRNA appeared to be expressed specifically in the kidney but not in the liver (Fig. 3). The uptake studies of methotrexate by use of monolayers of the rat OAT-K1 transfectant cells indicated clearly that the transporter is localized functionally at the basolateral membranes but not at the apical membranes (Fig. 5). Each anionic drug examined in the uptake studies, such as taurocholate, p-aminohippurate, prostaglandin E₂, and glutathione S-conjugate leukotriene C₄, a substrate with the highest affinity for multidrug resistance protein (MRP) (24), was not taken up by the rat OAT-K1-expressing cells (Fig. 4). Although taurocholate and p-aminohippurate inhibited weakly the methotrexate transport (Fig. 7), both anions were not substrates for the rat OAT-K1. Therefore, the rat OAT-K1 has substrate specificity that is distinct from that of the oatp, PGT, and MRP and of the typical renal p-aminohippurate transporter. These findings suggest that the OAT-K1 mediates methotrexate uptake across the basolateral membranes in the renal tubules and is functionally independent of the systems known to transport other anionic drugs.

Methotrexate also undergoes biliary excretion by the liver. Horne and Reed (25) reported that methotrexate uptake into rat liver basolateral membrane vesicles is a carrier-mediated process, is independent of imposed Na⁺ or H⁺ gradients, is electrogenic, and is inhibited by various structurally unrelated anions and the folate analog. Although the rat oatp would be

![Comparison of the deduced amino acid sequences (A) and hydrophy plots (B) between rat OAT-K1 and rat oatp. A, conserved residues between two transporters are boxed. Putative membrane-spanning domains are aligned over the sequence with numbers (M1-M12), and potential N-linked glycosylation sites are indicated by stars. Potential protein kinase A phosphorylation sites (▲) and protein kinase C phosphorylation sites (●) are indicated. B, Kyte and Doolittle (17) hydrophy plots with a window of 13 amino acid residues. Numbers between plots indicate putative membrane-spanning regions.](image-url)
functional member of the liver organic anion transporters, whether the rat oatp recognizes and transports methotrexate remains unknown. The present findings that methotrexate is accumulated extensively in both the kidney and liver in vivo demonstrate the involvement of high affinity transport system(s) for methotrexate clearance at the basolateral membranes of these organs. The functional characterization of the rat OAT-K1 suggests that this transporter contributes to efficient basolateral uptake of methotrexate in the kidney but not in the liver. Further studies are needed to define a role of the rat oatp and OAT-K1 in the hepatic and/or renal elimination of anionic drugs.

In the present study, we observed that the structure analog folate has a potent inhibitory effect on the methotrexate uptake and is transported by the rat OAT-K1-expressing cells. A specific transporter for folate in rat intestinal basolateral membranes which is electroneutral and Na⁺-independent has been reported to exist (26). The rat OAT-K1 is unlikely an intrinsic folate transporter, because the OAT-K1 mRNA expression is not detected in the small intestine. The finding that the OAT-K1-mediated methotrexate uptake was inhibited by structurally unrelated organic anions, such as sulfobromophthalein, taurocholate, and DIDS suggest that the OAT-K1 is a multi-specific anion transporter involved in renal detoxification process. Furthermore, the rat OAT-K1 shows no significant amino acid identity and no structural homology with the intrinsic folate transporter proteins (27, 28). It should be further studied whether the rat OAT-K1 participates physiologically in the renal handling of folate.

Methotrexate accumulation in the LLC-OAT-K1 cells was unaffected in both the Na⁺- and Cl⁻-free incubation medium, suggesting that methotrexate transport is not mediated by either a methotrexate/Cl⁻ exchanger or a Na⁺-coupling process. Na⁺-independent transport system as for the case of rat oatp and PGT. Furthermore, the incubation temperature at
4 °C led a marked decrease in the methotrexate transport, whereas the condition in which the cellular ATP was depleted caused only a small depression of the methotrexate transport (Table I). These findings suggest that the process of OAT-K1-mediated methotrexate uptake might be a facilitated transport process but not a secondary active transport process. Precise transport mechanisms including its coupling with other ions of the OAT-K1 should be further studied.

In conclusion, cDNA encoding a novel member of organic anion transporter proteins, OAT-K1, was isolated from the kidney of rats. Predominant expression of the OAT-K1 in the kidney among rat tissues, its functional properties, and localization at the basolateral membranes of the transfected cell line suggest that the OAT-K1 contributes to renal clearance of methotrexate from the blood.

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FIG. 6. Concentration dependence of [3H]methotrexate accumulation by LLC-OAT-K1 cells. [3H]Methotrexate accumulation was measured at various concentrations (0.05–10 μM) for 15 min at 37 °C added to the basolateral side (2 ml, pH 7.4) of the monolayers in the absence (○) and presence (□) of 1 mM unlabeled methotrexate. Unlabeled incubation medium was added to the apical side (2 ml, pH 7.4). After incubation, the radioactivity of solubilized cells was determined. Broken line represents the specific component of methotrexate accumulation. Each point represents the mean ± S.E. of three monolayers.

FIG. 7. Effect of various anionic drugs on [3H]methotrexate accumulation by LLC-OAT-K1 cells. [3H]Methotrexate accumulation was measured at various concentrations (0.05–10 μM) for 15 min at 37 °C added to the basolateral side (2 ml, pH 7.4) of the monolayers in the absence (○) and presence (□) of 1 mM unlabeled methotrexate. Unlabeled incubation medium was added to the apical side (2 ml, pH 7.4). After incubation, the radioactivity of solubilized cells was determined. Broken line represents the specific component of methotrexate accumulation. Each point represents the mean ± S.E. of three monolayers.

**TABLE I**

Effect of Na+, Cl−, temperature, and ATP depletion on methotrexate accumulation in LLC-OAT-K1 cells.

| Treatment            | [3H]Methotrexate fmol/mg protein/15 min % control | Control |
|---------------------|-----------------------------------------------|---------|
| Cl−-free            | 155.2 ± 23.4                                  | 100     |
| Na+-free            | 144.0 ± 3.3                                   | 119     |
| 4 °C                | 54.4 ± 4.1*                                   | 45      |
| ATP depletion       | 103.3 ± 1.6*                                  | 81      |

* p < 0.001, significant difference from control.

**TABLE II**

Tissue distribution of [3H]methotrexate after the intravenous bolus administration.

| Tissues          | Concentrationa | Ratios | Concentrationb | Ratios |
|------------------|----------------|--------|----------------|--------|
| Plasma           | 1.1 ± 0.0      | 1      | 0.2 ± 0.0      | 1      |
| Brain            | 0.3 ± 0.1      | 1      | 0.2 ± 0.1      | 1      |
| Heart            | 1.4 ± 0.0      | 1      | 0.6 ± 0.0      | 3      |
| Lung             | 3.0 ± 0.1      | 1      | 1.1 ± 0.0      | 6      |
| Liver            | 29.3 ± 0.6     | 1      | 24.7 ± 1.9     | 100    |
| Small intestine  | 3.1 ± 0.5      | 1      | 4.2 ± 0.4      | 21     |
| Spleen           | 1.2 ± 0.1      | 1      | 0.9 ± 0.0      | 5      |
| Kidney cortex    | 91.2 ± 7.5     | 83     | 184.6 ± 2.3    | 923    |
| Kidney medulla   | 60.7 ± 2.1     | 55     | 116.8 ± 3.3    | 584    |

a fmol/ml for plasma and pmol/g for tissues.

b [3H]Methotrexate concentration ratios are calculated from tissue/plasma concentration.
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