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Distribution of mRNA of a Na^+-independent neutral amino acid transporter cloned from rat kidney and its expression in mammalian tissues and *Xenopus laevis* oocytes

(tryptophan/system L transport)

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ABSTRACT **The Na^+-independent neutral amino acid transporter (NAA-Tr) that we had previously cloned from rat kidney has been investigated with respect to its distribution in mammalian tissues and cells. By Northern blot analysis and RNase protection assay, a 2.4-kilobase (kb) mRNA in rat intestine was found to be identical to that in rat kidney. Of the other rat tissues examined, only brain and heart were found to contain mRNAs related to kidney NAA-Tr by Northern assay. However, these were larger (≈5 and ≈7 kb). Mouse and rabbit kidney also contain mRNAs of 2.4 kb that exhibited a high degree of homology with rat kidney NAA-Tr. Of the several cultured cells investigated that demonstrated considerable Na^+-independent neutral amino acid transport activity, only human colon carcinoma (Caco) cells were positive by Northern assay. The failure to detect NAA-Tr mRNA in many cells and tissues that carry out Na^+-independent transport indicates that unrelated transporters must also exist. Cells and tissues that were negative with respect to rat kidney NAA-Tr as well as those that were positive transported leucine and tryptophan equally well. However, when mRNA from the same cells and tissues was expressed in oocytes, in all cases tryptophan was transported far less efficiently than leucine. This defect in tryptophan transport is apparently due to aberrant expression of neutral amino acid transporters in general in *Xenopus* oocytes.**

We recently reported the cloning and sequencing of a cDNA encoding a sodium-independent L-type neutral amino acid transporter (NAA-Tr) from rat kidney (1). When the complementary RNA (cRNA) transcribed from this clone was injected into *Xenopus laevis* oocytes, transport activity was expressed that was similar to that of the well-characterized L-type system (see ref. 2 and references therein) with respect to leucine and phenylalanine uptake, but it differed in other respects, most notably in that tryptophan was transported very poorly. The possibility that we had cloned a variant of the L transporter led us to investigate the distribution of NAA-Tr mRNA in various mammalian tissues and cells. We also compared tryptophan transport in many cells and tissues with the transport of tryptophan in oocytes injected with either NAA-Tr cRNA or mRNA isolated from the same tissues and cells.

MATERIALS AND METHODS

Materials. ^3H-labeled amino acids, [α-^{32}P]dCTP, and [α-^{32}P]CTP were from Amersham. 2-Aminobicyclo[2,2,1]-heptane-2-carboxylic acid (BCH) was purchased from Aldrich. 3-(4-Aminonaphthyl)alanine (*p*-ANA) was custom synthesized by Regis (Morton Grove, IL). Plasmid pSV·SPORT1 (*Sal* I/*Not* I cut) was from Bethesda Research Laboratories. The Riboprobe RNA transcription kit and RNA polymerases were from Promega. The RNase protection assay kit (RPA II) was from Ambion (Austin, TX). The RNaid kit was from Bio 101 (La Jolla, CA). Restriction enzymes were from Boehringer Mannheim and Promega. Tissues were obtained from Wistar rats, $(B6C3)F_1$ mice, and New Zealand White rabbits. Oocyte-positive *X. laevis* females from Nasco (Fort Atkinson, WI) were maintained at 19°C. Cell lines were obtained from American Type Culture Collection except for human Caco-2 cells, which were kindly supplied by Carole Bailey (Hoffmann–LaRoche, Nutley, NJ).

Northern Blot Analysis. Total RNA was extracted from the sources indicated by the method of Chomczynski and Sacchi (3) (the reagent RNazol was purchased from Biotecx Laboratories, Houston), and poly(A)$^+$ RNA was purified by affinity chromatography on oligo(dT)-cellulose columns (BRL). RNA was fractionated on 1.2% agarose/2.2 M formaldehyde gels and blotted onto nitrocellulose membranes. A 2.16 kilobase (kb) *Mlu* I/*Bsu*36I cDNA fragment from the plasmid pSPORT/NAA-Tr (1) was labeled with ^{32}P by using an oligolabeling kit (Pharmacia). Hybridizations and washings were carried out essentially as described (4) except that hybridizations were done at 37°C and the blots were washed successively at moderate [50°C with 1× standard saline citrate (SSC)/0.1% SDS] and high (67°C with 0.1× SSC/0.1% SDS) stringencies. A 1.3-kb *Pst* I fragment of glyceraldehyde-3-phosphate dehydrogenase cDNA, kindly provided by R. Narayanan (Roche Research Center), was labeled with ^{32}P and used for hybridization on regenerated RNA blots as an internal standard.

RNase Protection Assay. The three probes used in this assay were constructed in the plasmid vector pSPORT1 (BRL). Probe 3 is a full-length cDNA clone containing the nucleotide sequence 1–2305 of NAA-Tr. Probe 2 contains the nucleotide sequence 1–407 of the cDNA. Probe 1 represents the sequence 287–407 of the cDNA, which codes for the first putative membrane-spanning domain and the succeeding 14 amino acid residues of the first extracellular loop of NAA-Tr (1). The plasmids were linearized with *Sma* I and the ^{32}P-labeled antisense Riboprobes were transcribed from an SP6 promoter in the presence of [α-^{32}P]CTP using the Riboprobe Gemini transcription system (Promega). The RNase protection assay was performed as described (5). Total RNA (10–20 μg) was hybridized with 10^4–10^5 cpm of ^{32}P-labeled Riboprobes at 42°C overnight and the digestion was carried out with 1:100 diluted RNase A/RNase T1 mixture (RPA II kit; Ambion) at 37°C for 2 hr. The samples were electrophoresed on a 6% denaturing acrylamide gel and autoradiographed at

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Abbreviations: NAA-Tr, rat kidney neutral amino acid transporter; cRNA, complementary RNA; BCH, 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid; *p*-ANA, 3-(4-aminonaphthyl)alanine.

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−70°C. The Riboprobe 1, 2, and 3 transcripts contain 176, 478, and 2380 nucleotides, respectively. Fully protected fragments of Riboprobes 1, 2, and 3 contained 120, 407, and 2305 nucleotides, respectively. A rat β-actin cDNA [120 base pairs (bp)] subcloned in pBluescript II (KS+) vector (Stratagene) (kindly provided by P. McKinnon of the Roche Institute) was transcribed from a T7 promoter and used as an internal control for the RNase protection assay.

Uptake of L-Leucine and L-Tryptophan by Cultured Cells. Cells were cultured to confluence in appropriate medium at 37°C under a water-saturated 5% CO_2/95% air atmosphere. The harvested cells were washed three times in phosphate-buffered saline (PBS) and 0.1 ml of the cell suspension (10^5–10^6 cells) was incubated with 50 μM ^{3}H-labeled L-leucine or L-tryptophan at 30°C for 1 min in the absence or presence of inhibitors (unlabeled L-leucine or L-tryptophan at 0.5 mM, BCH at 2 mM, and *p*-ANA at 1 mM). The uptake of radiolabeled amino acids into cells was determined essentially as described by Segal and Lichtman (6). Briefly, the reactions were terminated by rapidly transferring the cells onto a 0.5-ml cushion of dibutylphthalate/corn oil mixture (4:1; vol/vol) in a 1.5-ml Eppendorf tube and centrifuging for 10 sec. The tip of the tube containing the cell pellet was cut off. The cell pellet was dispersed in 1 ml of PBS and lysed with 10 μl of 10 M NaOH; radioactivity was determined by liquid scintillation counting. Corresponding controls were carried out by incubating the cells at 0°C and these values were subtracted from the uptake at 37°C. Amino acid uptake into transfected COS-7 cells was carried out by a plate assay method (7). Briefly, cells on a 12-well tissue culture plate were incubated with PBS-GMC (0.01 M phosphate/0.15 M NaCl/5.6 mM D-glucose/0.49 mM $MgCl_2$/0.68 mM $CaCl_2$, pH 7.4) at 37°C for 40 min with one change of the PBS-GMC to deplete internal amino acid pools. Then 0.5 ml of fresh PBS-GMC containing 50 μM ^{3}H-labeled amino acid with or without inhibitors was added to each well and incubation was continued at 37°C for up to 60 min. The reaction was stopped by adding PBS-GMC (previously cooled to 0°C) and the cells were rinsed three times with the cold PBS-GMC. The washed cells were solubilized with 0.05% sodium deoxycholate/0.1 M NaOH, and aliquots of the cell lysate were removed for scintillation counting or for protein assay with the Bradford reagent (8). Uptake into *X. laevis* oocytes was performed as described (1). The cRNA of rat kidney NAA-Tr was transcribed from the corresponding cDNA clone and purified by using the RNaid RNA purification kit (Bio 101).

Amino Acid Uptake by Rat Kidney Slices. Rat kidney slices (freshly prepared from 24-hr fasted adult rats; ≈80 mg wet weight and ≈400 μm thick) were washed thoroughly at 37°C with Krebs solution (5 mM $CaCl_2$/3.6 mM $MgSO_4$/118 mM NaCl/4.7 mM KCl/14 mM glucose/25 mM $NaHCO_3$/1.8 mM KH_2PO_4, pH 7.3). To monitor transport, they were incubated at 37°C for 10 min in 1 ml of the Krebs solution containing either 1 mM [^{3}H]leucine or 1 mM [^{3}H]tryptophan in the absence or presence of the competing amino acids (20 mM). After incubation, the slices were immediately rinsed three times with 1-ml aliquots of cold Krebs solution. The slices were briefly drained on filter paper and then homogenized in 0.75 ml of cold 0.2 M HCl and deproteinized by adding 250 μl of 30% trichloroacetic acid to the homogenate followed by centrifugation. An aliquot of the protein-free supernatant was taken for scintillation counting. Corresponding controls were run by incubating the slices at 0°C and the values were subtracted from values obtained by incubating at 37°C. Uptake is expressed as nmol of amino acid transport per hr per mg wet weight of tissue; each value represents the average of two experiments.

Expression of NAA-Tr in COS-7 Cells. A *Sal* I/*Not* I fragment containing the full-length cDNA was released from pSPORT/NAA-Tr (1) and subcloned into pSV·SPORT1 (*Sal* I/*Not* I cut) mammalian expression vector. The plasmid was purified by using a Qiagen column (Qiagen, Chatsworth, CA). COS-7 cells (10^5 cells per well) cultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum were seeded into a 12-well tissue culture plate 24 hr before being transfected with the plasmid DNA (200 ng per well) by the DEAE-dextran method (9). Plate assays of amino acid uptake were performed 3 days after transfection as described above.

RESULTS AND DISCUSSION

Distribution of NAA-Tr and Related mRNAs. Northern transfer and hybridization analysis using the NAA-Tr cDNA probe revealed, as reported (1), the presence of a 2.4-kb mRNA in rat kidney (Fig. 1). An mRNA of identical size, which hybridizes even at high stringency, was also detected in rat intestine (jejunum) (Fig. 1*B*). Further evidence for the identity of the 2.4-kb mRNAs detected in rat kidney and intestine with the NAA-Tr cRNA was obtained by RNase protection assay (Fig. 2*A*). Additional (but less abundant) mRNAs of about 5 and 7 kb in rat kidney and intestine hybridized at moderate stringency (Fig. 1*A*). Interestingly, 5-kb transcripts were also detected in rat brain and, to a lesser extent, in heart when hybridization was carried out at moderate stringency (Fig. 1*A*). The significance of these additional cross-reacting species is at present not known. However, there was no protection of the NAA-Tr cRNA Riboprobes when RNase protection assays were performed with mRNAs from rat brain and heart (Fig. 2*A*). This indicates

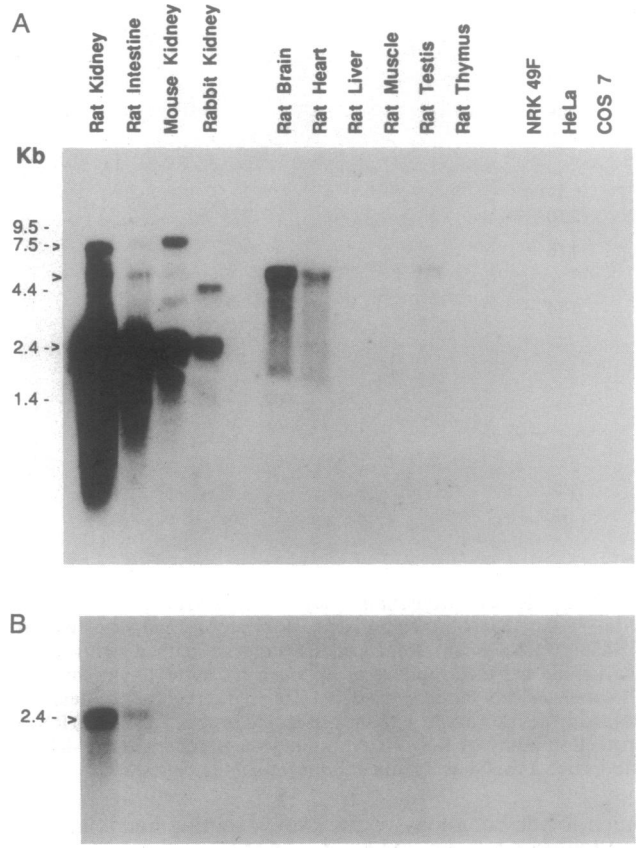

FIG. 1. Northern analysis of mRNAs isolated from various tissues and cells. mRNAs (2 μg) were fractionated on a 1.2% agarose/2.2 M formaldehyde gel and immobilized on a nitrocellulose membrane. (*A*) Blot was hybridized with the ^{32}P-labeled rat kidney NAA-Tr cDNA probe and washed at moderate stringency (with 1× SSC/0.1% SDS at 50°C). (*B*) Same blot as in *A* washed at high stringency (with 0.1× SSC/0.1% SDS at 67°C). RNA standards are on the left.

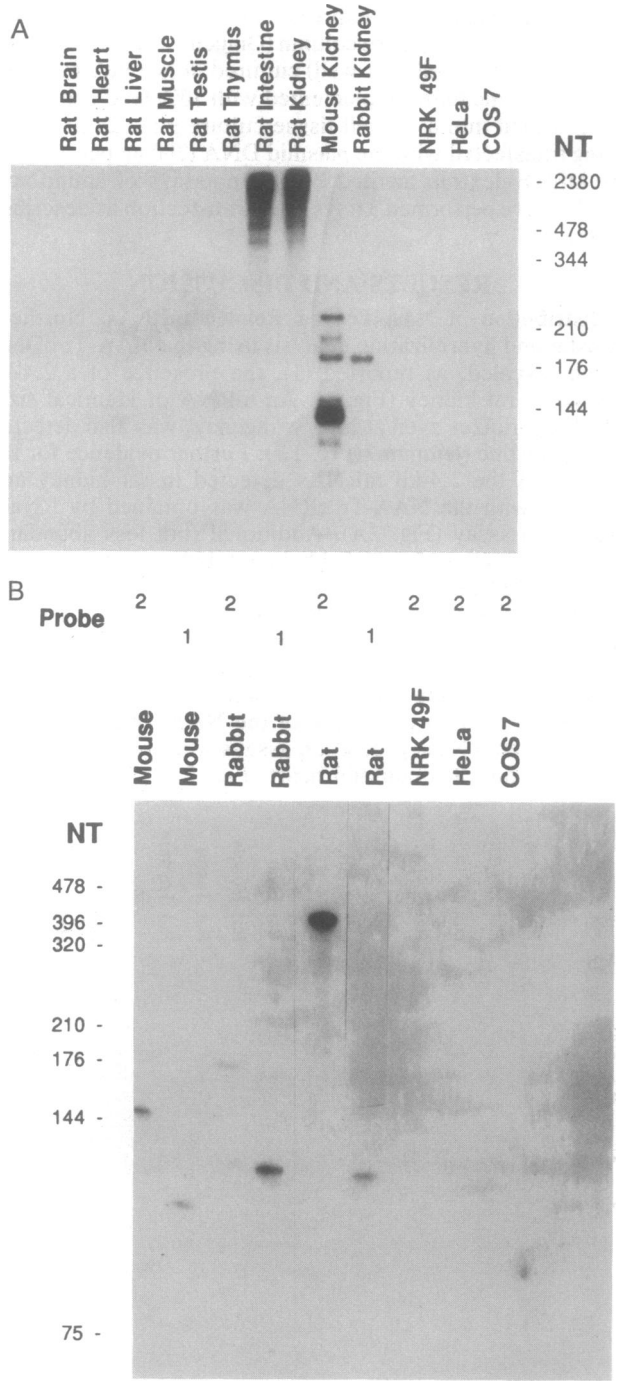

FIG. 2. RNase protection assay. Total RNAs were hybridized with ^{32}P-labeled NAA-Tr antisense Riboprobes and digested with RNase A/RNase T1. Reaction mixtures were then analyzed by acrylamide gel electrophoresis followed by autoradiography. NT, ^{32}P-labeled 1-kb ladders used as size markers (nucleotides). (*A*) RNase protection assay with the full-length probe (2.3 kb). (*B*) RNase protection assay of RNAs from kidneys and cells with ^{32}P-labeled Riboprobe 1 (120 bases) and Riboprobe 2 (407 bases).

much lower homology of the cross-reacting bands in brain and heart with kidney NAA-Tr mRNA.

Like rat kidney, mouse and rabbit kidneys also exhibited a 2.4-kb mRNA that hybridized with the NAA-Tr cDNA probe at moderate stringency (Fig. 1*A*). Human kidney and pancreas also yielded 2.4-kb bands at moderate stringency (data not shown). Based on the size of protected fragments of Riboprobe 3 (full-length probe) (Fig. 2*A*), the identity between rat and mouse mRNA was estimated to be at least 70%. Identity between rat and rabbit mRNA is apparently lower. Interestingly, when the shorter probe (Riboprobe 1), which codes for the first transmembrane domain of rat kidney NAA-Tr, was used in the RNase protection assay, protected fragments of about the same size (≈120 bp) were seen with rat, mouse, and rabbit kidney mRNAs (Fig. 2*B*). These findings predict that the first transmembrane domain and the succeeding 14 amino acid residues are highly conserved across species.

All mammalian cells and tissues have been shown to possess Na^+-independent system L neutral amino acid transporter activity (2). However, NAA-Tr mRNA could not be detected in several rat tissues (e.g., liver, skeletal muscle, thymus, and testis). We also investigated a number of established cell lines that exhibit relatively high system L transport. Transport of leucine and tryptophan into these cells is shown in Table 1 and is compared to their uptake into rat kidney slices. However, in spite of the high transport rates shown by these cells, NAA-Tr mRNA could not be detected in NRK, HeLa, and COS-7 cells by either Northern blot analysis or RNase protection assays (Figs. 1 and 2). This indicates that system L transport in such cells is encoded by mRNAs with little or no homology with the rat kidney NAA-Tr mRNA. It should be noted that we previously showed that a 2.4-kb mRNA in Caco-2 cells (derived from human colon carcinoma) hybridized at moderate stringency with NAA-Tr cDNA (1).

Characteristics of Neutral Amino Acid Transport Expressed in Oocytes and in Mammalian Cells. As shown in Table 1, leucine and tryptophan are both transported efficiently and at similar rates into the mammalian cells we examined and into rat kidney slices. Furthermore, BCH [a paradigm substrate for system L transport (2, 10)] and *p*-ANA (11) are potent inhibitors of both leucine (Table 1) and tryptophan (data not shown) transport into these cells. In addition, tryptophan strongly inhibits leucine uptake and leucine has a similar effect on tryptophan uptake (data not shown). In contrast [as reported previously (1)], when cRNA transcribed from the cloned rat kidney NAA-Tr cDNA was injected into *Xenopus* oocytes, the expressed transport exhibited unusual characteristics. Thus, although the apparent K_m values for leucine and phenylalanine for NAA-Tr expressed in oocytes were similar to those found in mammalian cells [≈20 μM (1, 2)], BCH and *p*-ANA [and tryptophan (data not shown)] were relatively poor inhibitors of leucine transport (and of phenylalanine transport) mediated by NAA-Tr in oocytes (Table 2). The V_{max} for tryptophan transport by NAA-Tr in oocytes was ≈10% that of leucine or phenylalanine and the apparent K_m for tryptophan was ≈250 μM or about an order of

Table 1. Transport of L-leucine and L-tryptophan into mammalian cells

Cell or tissue	Uptake, nmol per min per 10^6 cells		Ratio Trp/ Leu	% inhibition of L-leucine uptake	
	Leu	Trp		By BCH	By *p*-ANA
Rat kidney slices	0.4*	0.7*	1.75	ND	ND
Caco-2	4.4	5.4	1.23	100	100
HeLa	1.1	1.1	1.00	100	99
COS-7	1.5	1.7	1.08	85	98
NRK 49F	3.4	3.6	1.05	85	99

Uptake of ^{3}H-labeled amino acids into rat kidney slices and into mammalian cells, in the absence or presence of inhibitors (2 mM BCH; 1 mM *p*-ANA), was determined as described. Each value is an average of three independent experiments. The four cell lines used are Caco-2, human colon carcinoma; HeLa, human cervical carcinoma; COS-7, monkey kidney (fibroblast-like); and NRK 49F, rat kidney fibroblast. ND, not determined.
*nmol per hr per mg wet weight.

Table 2. Expression of L-leucine and L-tryptophan transport in *Xenopus* oocytes

RNA source	Uptake, pmol per hr per oocyte		Ratio Trp/ Leu	% inhibition of L-leucine uptake	
	Leu	Trp		By BCH	By *p*-ANA
mRNA					
Rat kidney	170	24	0.14	15	22
Mouse kidney	146	6	0.04	15	20
Rabbit kidney	160	18	0.11	17	21
Caco-2	58	4	0.07	16	20
HeLa	27	<1	<0.04	30	39
COS-7	8.1	1.6	0.2	18	ND
cRNA					
NAA-Tr	1674	81	0.05	17	19

Oocytes were injected either with mRNA (50 ng per oocyte) from the tissue or cells indicated or with NAA-Tr cRNA (10 ng per oocyte). Uptake of ^{3}H-labeled amino acids (50 μM each) at 20°C was measured 3 days after injection in the absence or presence of inhibitors (2 mM BCH; 1 mM *p*-ANA), as described (1). Values for uptake of ^{3}H-labeled amino acids into water-injected oocytes (15 and 12 pmol per hr per oocyte, respectively, for leucine and tryptophan) were subtracted from the corresponding uptake measurements on mRNA- or cRNA-injected oocytes. Each value is an average of independent measurements on six to eight oocytes. ND, not determined.

magnitude larger than the K_m for either leucine or phenylalanine (Table 3). These findings led us to conclude, initially, that the rat kidney NAA-Tr we had cloned was a subtype of system L transporter exhibiting a low preference for tryptophan (1). This conclusion seemed to be confirmed when the same anomalous transporter phenotype was found to be expressed in oocytes injected with mRNAs from mouse and rabbit kidneys and from human Caco-2 cells (Table 2), tissues that contain NAA-Tr mRNA related transcripts (Figs. 1 and 2). To our surprise, however, mRNAs from HeLa and COS-7 cells, which were negative with respect to NAA-Tr mRNA, when injected into *Xenopus* oocytes also expressed an L-type transporter that was again defective in tryptophan transport. This is in contrast to the transport characteristics observed by the same cells when they were intact (Table 1). Thus, the defect in tryptophan transport appears to be due to aberrant expression of L-type transporters in general in *Xenopus* oocytes. It may be noted that selective as well as incorrect expression of a number of membrane proteins in *Xenopus* oocytes have been noted by others (12). In particular, the catfish olfactory cyclic nucleotide gated ion channel when expressed in *Xenopus* oocytes exhibits an apparent K_m for cyclic nucleotides that is 10-fold higher than the apparent K_m of the native channel in olfactory sensory neurons (13).

Table 3. Kinetic parameters for transport of amino acids by rat kidney NAA-Tr

Amino acid	K_m, μM	Relative V_{max}
L-Phenylalanine	29	100
L-Leucine	22	97
L-Tryptophan	250	11

Xenopus oocytes were injected with 5 ng of NAA-Tr cRNA per oocyte. Three days after injection, amino acid uptake was determined as described (1). Concentrations of leucine and phenylalanine were varied from 10 to 100 μM and that of tryptophan was varied from 25 to 250 μM. Average V_{max} for uptake of phenylalanine was ≈2600 pmol per hr per oocyte. Although absolute values for V_{max} varied appreciably from experiment to experiment, the relative values were fairly constant. Accordingly, V_{max} values are reported relative to that of phenylalanine taken as 100. V_{max} values for leucine and tryptophan are expressed as percentage of V_{max} for phenylalanine. Data for leucine and phenylalanine were reported in a previous publication (1).

Attempts were made to express cloned renal NAA-Tr in COS-7 cells to determine whether the properties of the expressed transporter would be more typical of system L transporters. Although NAA-Tr mRNA was readily detected in the transfected cells by Northern blot analysis (data not shown), the basal endogenous rate of uptake of leucine and tryptophan by these cells was so high that expression of any additional uptake due to transfection with NAA-Tr cDNA was not readily detected. Interestingly, when total cellular RNA from transfected cells was injected into oocytes (75 ng per oocyte), there was an ≈9-fold increase in the rate of leucine transport compared to oocytes injected with RNA from mock-transfected COS-7 cells. This shows that NAA-Tr cDNA was indeed transcribed in transfected COS-7 cells. However, the transport expressed in oocytes from mRNA of transfected cells again exhibited characteristics similar to those shown by oocytes injected with the cloned NAA-Tr cRNA (i.e., weak inhibition by BCH and *p*-ANA and poor uptake of tryptophan).

The question regarding the functional characteristics of NAA-Tr in mammalian cells requires further study. An important conclusion from the present experiments is that the NAA-Tr that we have cloned appears to be restricted to epithelial cells, such as those of the kidney and intestine, and may therefore be involved in transcellular transport of amino acids across such cells (i.e., from the intestinal lumen into the blood or reabsorption by the renal tubules). Another conclusion from the present experiments is that NAA-Tr is not the only system L-type transporter in mammalian cells. Since all cells and tissues must transport nutritionally essential neutral amino acids, failure to detect NAA-Tr mRNA, even under relatively low stringency conditions, in cells and tissues that carry out L-type transport, indicates that other, unrelated, types of system L transporters must also exist.

Note Added in Proof. Our initial report on the cloning of a neutral amino acid transporter from rat kidney appeared in ref. 1. This report represents a continuation of that work. After the present paper was submitted, we became aware of a paper by Wells and Hediger (14), which reported cloning the identical cDNA from the same tissue, rat kidney. Surprisingly no reference was given to our earlier paper. In their report, they observed transport of basic as well as neutral amino acids in *Xenopus* oocytes injected with the corresponding cRNA. We have confirmed Wells and Hediger's finding that our NAA-Tr transports basic amino acids as well as neutral amino acids.

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