

Expression Cloning of a Human Renal cDNA That Induces High Affinity Transport of L-Cystine Shared with Dibasic Amino Acids in *Xenopus* Oocytes*

(Received for publication, November 12, 1992, and in revised form, March 8, 1993)

Joan Bertran^{‡§¶}, Andreas Werner^{§¶}, Josep Chillarón^{‡§¶}, Virginia Nunes^{**}, Jürg Biber[¶],
Xavier Testar[‡], Antonio Zorzano[‡], Xavier Estivill^{**}, Heini Murer[¶], and Manuel Palacín^{‡‡}

From the [‡]Departament de Bioquímica i Fisiologia, Universitat de Barcelona, E-08028 Barcelona, Spain, the [¶]Physiologisches Institut, Universität CH-8057 Zürich, Switzerland and the ^{**}Departament de Genètica Molecular, Institut de Recerca Oncològica, E-08907 Barcelona, Spain

A renal cDNA clone (rBAT) that induces system b⁰⁺-like amino acid transport activity in *Xenopus* oocytes has recently been isolated (Bertran, J., Werner, A., Moore, M. L., Stange, G., Markovich, D., Biber, J., Testar, X., Zorzano, A., Palacín, and Murer, H. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 5601-5605). Here we show the isolation of a cDNA clone by screening a human kidney cortex cDNA library for expression of sodium-independent transport of L-[³H]arginine in *Xenopus* oocytes. The cRNA of this clone induces in oocytes, in addition to the uptake of L-arginine, that of L-[³⁵S]cystine and L-[³H]leucine. Expressed uptake of these amino acids is mutually cis-inhibitable by the other 2 amino acids. Expressed uptake of L-cystine is saturable and shows an apparent K_m in the micromolar range. All these characteristics resemble induction of system b⁰⁺ related to rBAT in the oocytes. Human rBAT mRNA (~2.5 kilobases) is found in kidney, small intestine (*i.e.*, jejunum), pancreas, and liver. Human kidney poly(A)⁺ RNA (mRNA) induces sodium-independent uptake of L-cystine, L-arginine, and L-leucine in *Xenopus* oocytes. Hybrid depletion with an antisense oligonucleotide of the isolated clone greatly prevents (80-97%) human kidney mRNA-dependent induction of the uptake of these amino acids (*i.e.* L-cystine, L-arginine, and L-leucine). The isolated clone (2304 base pairs in length) contains a poly(A) tail and encodes a predicted 78.8-kDa protein which is 85 and 80% identical to the rabbit and rat rBAT, respectively. This predicted protein corresponds to a membrane glycoprotein, and contains six potential N-glycosylation sites which might be functional in the oocyte: [³⁵S] methionine labeling of oocytes shows a specific band of 94 kDa in crude membranes of these human cRNA-

injected oocytes; treatment of these oocytes with tunicamycin shifts the cRNA-specific translation product to approximately 72 kDa. We conclude that we have isolated a functional cDNA corresponding to human rBAT. The isolation of this human cDNA would lead to the study of the possible involvement of rBAT in human hyperaminoacidurias.

Recently, two highly homologous kidney cortex cDNA clones (named rBAT-1; *i.e.* b⁰⁺ amino acid transporter-related, for the rabbit cDNA and D2 for the rat cDNA) have been isolated, which upon *in vitro* transcription and capping complementary RNA (cRNA), and injection into *Xenopus laevis* oocytes induce system b⁰⁺-like amino acid transport activity (1-2). The rBAT-induced activity (also D2) carries, with high affinity, L-dibasic, some L-neutral amino acids (*e.g.* preferentially L-leucine, L-methionine, and L-phenylalanine) and L-cystine in oocytes (1-5). Tate and co-workers (4, 5) isolated a cDNA clone, identical to rat kidney D2 cDNA, that named NAA-Tr¹ (*i.e.* neutral amino acid transporter), because they initially reported that it was responsible for only neutral amino acid transport expression in oocytes. More recently, a second rabbit rBAT clone (rBAT-2) has been isolated from a rabbit kidney cortex cDNA library. This rBAT-2 cDNA corresponds, with an alternative polyadenylation, to the longer rBAT transcript seen in Northern blots, and it is responsible for the expression of the same transport activity for dibasic and neutral amino acids and cystine as rBAT-1 (also D2) in oocytes (6).

rBAT messenger RNA (also D2 mRNA) is found mainly in kidney and intestinal mucosa, both in rabbit and rat (1-2). In keeping with this, hybrid-depletion experiments of renal and intestinal mRNA with rBAT (also D2) antisense oligonucleotides showed functionality of rBAT mRNA when tested for expression of system b⁰⁺-like activity (*i.e.* L-arginine, L-leucine, and L-cystine uptake) in oocytes (2-3). Renal proximal tubular and small intestinal epithelial cells are involved in vectorial fluxes of different groups of amino acids. The basolateral membranes of proximal tubular cells are believed to contain amino acid carriers that are similar to those described for nonpolarized cells, whereas in the brush-border membranes additional transport systems are present (7). Among the latter, a sodium-independent transport system for neutral and dibasic amino acids, with a transport activity similar to

* This work was supported in part by Dirección General de Investigación Científica y Técnica Research Grant PB90/0435 (to M. P.), Swiss National Science Foundation Grant 32-30785-91 (to H. M.), and Fondo de Investigaciones Sanitarias Grant 92/0885E (to V. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L11696.

§ These authors have contributed equally to this study.

¶ Recipients of predoctoral fellowships from the Ministerio de Educación y Ciencia, Spain, and the Comissió Interdepartamental de Ciència i Innovació Tecnològica from Catalonia, Spain.

‡‡ To whom correspondence should be addressed: Dept. de Bioquímica i Fisiologia, Universitat de Barcelona, Avda. Diagonal 645, E-08028 Barcelona, Spain. Fax: 34-3-4021559.

¹ The abbreviations used are: NAA, neutral amino acid transport; kb, kilobase; PAGE, polyacrylamide gel electrophoresis.

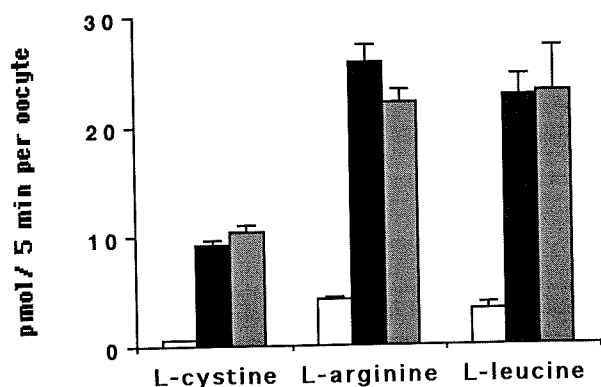


FIG. 1. Amino acid transport by water and human rBAT cDNA-injected oocytes. *Xenopus* oocytes were injected with 50 nl of water alone or containing 2.5 ng of synthetic RNA from the isolated cDNA clone (cRNA). Three days later, the uptake of the indicated amino acids, at a concentration of 50 μ M, was determined for 5-min (cystine and leucine) or 10-min (arginine) incubations. Each bar is the mean \pm S.E. of the uptake values in water (open bars) and in human (solid bars) or rabbit (shaded bars) rBAT cDNA-injected oocytes measured in 6–8 oocytes.

that of system b^{o+}, as first defined in mouse blastocysts (8), has been described (9–11).

Human cystinuria is a disorder of amino acid transport affecting the epithelial cells of the renal tubule and the gastrointestinal tract (12, 13). The defective transport of cystine and the dibasic amino acids is transmitted as an autosomal recessive trait (14). Studies with isolated brush-border membrane vesicles indicated several transport pathways for L-cystine: L-cystine is transported in renal membranes via a low K_m system, which is shared with dibasic amino acids, and via a high K_m system, which appears to be unshared (15–20); transport in jejunal vesicles involves the low K_m system (21). A defect in this shared transport activity has been demonstrated in biopsies of intestinal mucosa from cystinuric patients (22–24). This defect could correspond to the brush-border membrane b^{o+} activity. In this instance an intact system involved in neutral amino acid reabsorption (e.g. neutral brush-border (7)) would compensate for the reabsorption of neutral amino acids but not of dibasic amino acids and cystine. Interestingly, injection of rat and rabbit intestinal (e.g. jejunum) mRNA into oocytes results in the expression of L-cystine uptake via a low K_m system shared with dibasic amino acids and some neutral amino acids (i.e. L-leucine, L-phenylalanine) (3, 25). This uptake activity should correspond to the translation of rBAT messenger RNA, since it is almost (>90%) hybrid-depleted by an rBAT antisense oligonucleotide (3). Furthermore, L-cystine uptake expressed in oocytes injected with rat kidney cortex mRNA is almost completely inhibited by rBAT (D2) antisense hybrid-depletion (Ref. 2 and present paper; see "Results and Discussion"). In contrast, murine leukemia virus receptor and 4F2hc surface antigen, the two recently identified cDNAs that induce dibasic amino acid transport in oocytes, do not induce L-cystine uptake (26–29).

Taken together, these findings suggest that the rBAT gene might be involved in human cystinuria. As a first step towards testing this hypothesis we report here the isolation and characterization of a human kidney cortex cDNA that corresponds to the human rBAT.

EXPERIMENTAL PROCEDURES

Human Tissue Samples and Isolation of Total RNA and Poly(A)⁺ RNA—Human kidney cortex used to prepare the cDNA library was from a healthy kidney obtained by nephrectomy in an ureter tumor-

bearing patient. Human kidney cortex used to prepare poly(A)⁺ RNA for injections in *Xenopus* oocytes was obtained, after nephrectomy, from the healthy looking contralateral part of a hypernephroma-bearing kidney. For Northern blot analysis total pancreas was obtained from healthy pancreatic tissue from an organ donor. Jejunum was obtained from partial intestinal resection during pancreatectomy in a pancreatic carcinoma-bearing patient. Tissues were rapidly frozen in liquid nitrogen and RNA was extracted using the guanidinium-thiocyanate-phenol-chloroform method as described (30). Oligo(dT)-cellulose for purification of poly(A)⁺ RNA was purchased from Boehringer Mannheim and used following the manufacturer's protocol.

Oocytes and Injections—*X. laevis* females were obtained from H. Kähler (Institut für Entwicklungsbiologie, Hamburg, Germany). Small clumps of oocytes were treated with collagenase D (Boehringer Mannheim) at 2 mg/ml in a Ca²⁺-free solution (ORII: 82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 10 mM Hepes/Tris, pH 7.5) twice for 90 min to remove the follicular layer. After thorough washing with ORII solution and modified Barth's solution (88 mM NaCl, 1 mM KCl, 0.82 mM MgSO₄, 0.4 mM CaCl₂, 0.33 mM Ca(NO₃)₂, 2.4 mM NaHCO₃, 10 mM Hepes/Tris, pH 7.5), the oocytes were kept in modified Barth's solution overnight at 18 °C. After this incubation period, healthy looking stage VI oocytes were injected with mRNA, cRNA, or water. RNA samples were dissolved in water at concentrations varying from 0.05 to 1 mg/ml. For hybrid depletion experiments, human mRNA (0.5 mg/ml) was denatured at 65 °C for 5 min in a solution containing 50 mM NaCl and 40 μ M of a 21-mer oligonucleotide complementary to the human (also rat) rBAT mRNA sequence (antisense; 5'-GAA CAG CAC CTC CTT GGG CAT-3') starting at base 245 (base 202 of the coding region) and further incubated at 42 °C for 30 min prior to injection. For hybrid depletion experiments with rat kidney mRNA, the same rBAT antisense as for human mRNA was used, whereas with rabbit kidney mRNA 20 μ M of a 17-mer oligonucleotide complementary to rabbit rBAT starting at bases 572–556 (antisense; 5'-GCT GCC AGC AGG TTC TC-3'; Ref. 1) was used, following the protocol described elsewhere (3). mRNA samples were injected into oocytes by using a semiautomatic injector (Inject+Matic-system, J. A. Gabay, Geneva). The volume injected was 50 nl. Oocytes were then incubated at 18 °C for 3–6 days in modified Barth's solution containing gentamycin sulfate (20 mg/liter).

cDNA Cloning—An unidirectional cDNA library was constructed using the SuperScript cloning system (Bethesda Research Laboratories) starting from a size-selected mRNA population (1.7–3.5 kb) isolated from human kidney cortex tissue. The methods of size fractionation of the mRNA have been described elsewhere (31). For screening, plasmids from pools of 1000 clones were isolated using a miniprep kit (Promega), linearized with the endonuclease NotI at their 3'-end, and transcribed *in vitro* using the T7 RNA polymerase in the presence of m⁷GpppG (see below). cRNA was dissolved in water at concentrations varying from 0.3 to 0.05 mg/ml and injected (50 nl) into oocytes. Three days after injection the uptake of L-[³H] arginine was assayed and compared to basal values of water-injected oocytes. Once a positive pool was obtained it was further subdivided until a single clone was isolated.

The sequencing of human rBAT cDNA was performed in both directions directly in pSport-1 (Bethesda Research Laboratories). To this end, we combined the use of oligonucleotides (Applied Biosystems 391 Synthesizer) and the generation of subclones in Bluescript KS⁻. DNA sequencing was carried out automatically (Applied BioSystems 370A Sequencer), using fluorescent dideoxy terminators and a thermocycling protocol as developed by Applied BioSystems Inc.

Synthesis of Transcripts from cDNA (cRNA)—Human rBAT containing plasmid was isolated using a miniprep kit (Promega). The plasmid, cloned in pSport-1 (BRL), was linearized by XbaI restriction endonuclease digestion and transcribed *in vitro*, using T7 RNA Polymerase (Promega) in the presence of m⁷GpppG (New England Biolabs), as described elsewhere (32). Briefly, 1–2 μ g of linearized plasmid DNA was incubated in 1 \times transcription buffer supplied with the enzyme (Promega), 0.5 mM ATP, CTP, UTP, and m⁷GpppG, 10 mM dithiothreitol, 0.1 mM GTP, 1 unit/ μ l of RNA-guard^R (Pharmacia LKB Biotechnology Inc.), and 30 units of T7 RNA Polymerase (Promega) in a final volume of 50 μ l. After 1 h, DNA was digested using 10 units of DNase I (Boehringer Mannheim) and 50 units of RNA-guard^R. RNA was extracted twice with phenol/chloroform (1:1) and precipitated with ammonium acetate and ethanol. The cRNA was finally resuspended in 10 μ l of water and an aliquot was quantified by absorbance at 260 nm and transcript integrity was checked on a 1% agarose/formaldehyde gel.

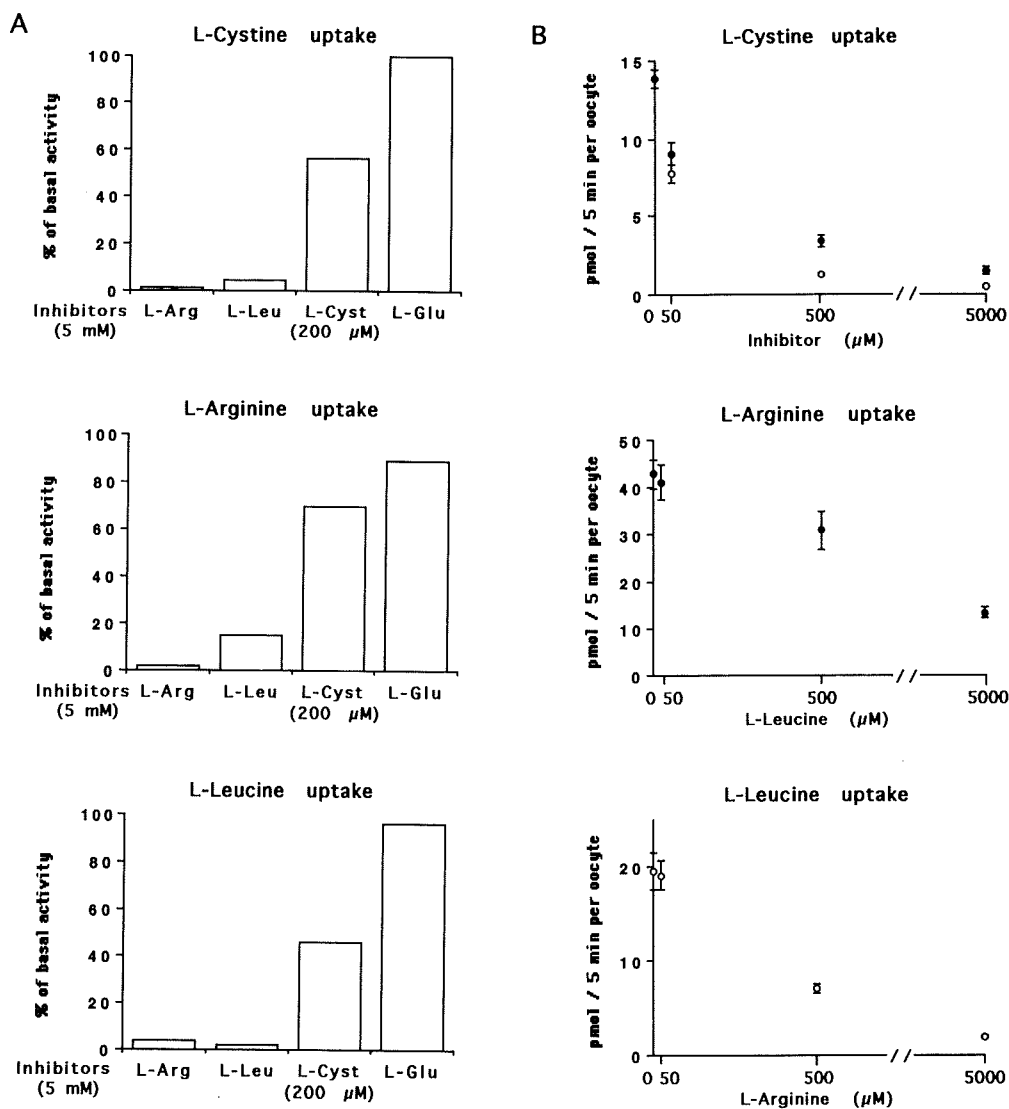


FIG. 2. Inhibition of human rBAT-induced transport activity by different amino acids. The human rBAT-induced transport activities (for L-cystine, L-arginine, and L-leucine), calculated by subtracting the uptake values obtained in water-injected oocytes from the uptake values in 2.5 ng of cRNA-injected oocytes, were tested for inhibition by the indicated amino acids. Inhibitors were added at a concentration of 5 mM except for L-cystine (200 μM) (A), and at different concentrations of L-arginine (open circles) and L-leucine (filled circles) (B). Uptake, measured at 50 μM substrate concentration 3 days after injection, was determined for 5-min (cystine and leucine) or 10-min (arginine) incubations. Each data point is the mean of values obtained in seven oocytes and is expressed as the residual percentage of uptake (A) and as the actual values of uptake (pmol/5 min/oocyte; mean ± S.E., $n = 6-8$ oocytes) (B), in a representative experiment. Another independent experiment showed identical results. All the inhibitions shown in A were statistically significant (Student *t* test; $p < 0.05$) with the exception of the cases in which L-glutamic acid was tested as an inhibitor.

Uptake Measurements—To measure the uptake of L-[³H]arginine, L-[³H]leucine, and L-[³⁵S]cystine (New England Nuclear Radiochemicals) 7–10 oocytes per individual data point were first washed for 30 s in solution A (100 mM choline chloride, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM Hepes/Tris, pH 7.5). To initiate uptake, this solution was replaced by 90 μl of solution A supplemented with the desired concentration of substrate, typically 50 μM at 10 μCi/ml. When L-cystine uptake was measured, 10 mM diamide was added to prevent the reduction of disulfide bonds. Inhibition by other amino acids was performed by their addition to the uptake solution. Uptakes were performed at 25 °C for 1 h when oocytes injected with poly(A)⁺ RNA were assayed, or for 5 min when clone cRNA-injected oocytes were used. After incubation, the uptake solution was removed and the oocytes were washed three times in 4 ml of ice-cold solution A supplemented with 5 mM cold substrate or 5 mM L-arginine + 5 mM L-leucine when L-cystine was measured. Immediately, each single oocyte was put into a scintillation vial, dissolved in 200 μl of 10% SDS, and the radioactivity counted after adding 3 ml of scintillation fluid.

Northern Blot Analysis—Total RNA from human tissues was

transferred to nylon membranes (Hybond N, Amersham) by capillary in 20 × standard saline citrate (SSC: 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) after size separation on a 1.2% agarose/formaldehyde gel. RNA was visualized with ethidium bromide to ensure that it was intact, and loaded in similar amounts to confirm proper transfer. In addition, a commercial blot (Clontech) containing 2 μg of highly pure poly(A)⁺ RNA from different human tissues was used. Blots were prehybridized in 5 × Denhardt's (1 × Denhardt's: 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin), 0.5% SDS, 5 × SSPE (1 × SSPE: 0.15 M NaCl, 1 mM EDTA, 10 mM NaH₂PO₄, pH 7.4), 100 μg/ml salmon sperm DNA and 50% formamide, and hybridized in the same solution containing 10% dextran sulfate. After hybridization, blots were washed in 2 × SSC for 10 min at room temperature and then twice for 20 min in 0.4 × SSC, 0.1% SDS at 55 °C. The cDNA probe was a 2.2-kb BamHI fragment lacking 148 base pairs at the 5'-end of the complete human rBAT cDNA purified from a 1% agarose gel using the GeneClean kit (BIO-101, La Jolla, CA). Probes were labeled with [³²P]dCTP (1.5 × 10⁶ cpm/ml) by random oligonucleotide priming (Boehringer Mannheim).

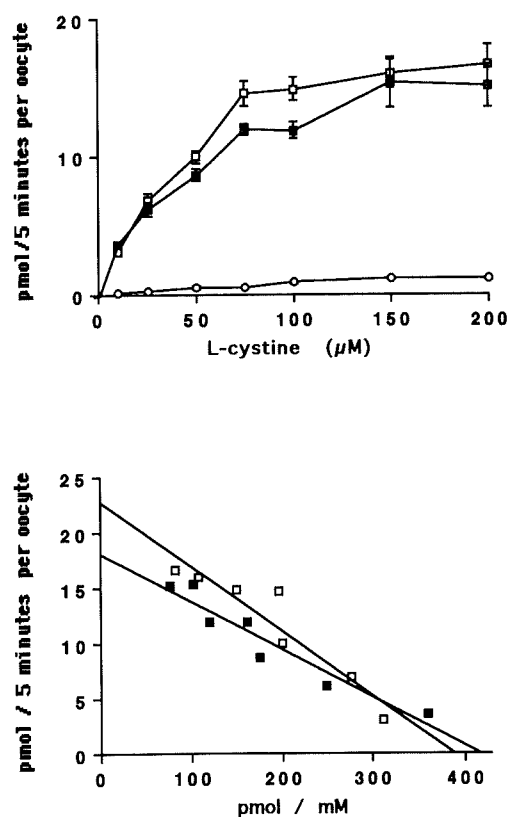


FIG. 3. Kinetic analysis of rBAT-induced transport of L-cystine. Oocytes were injected with water (○) or 2.5 ng of human (■) or rabbit (□) cRNA. Three days later, L-cystine uptake was determined, at different amino acid concentrations, for 5-min incubations. In the upper graph, data represent the uptake values in water-injected oocytes and the rBAT-induced transport activities (see legend to Fig. 2). Data are mean \pm S.E. from 6–8 oocytes in a representative experiment. When not visible, error bars are smaller than the symbol. A second independent experiment showed identical results. Eadie-Hofstee transformation of rBAT-induced transport of L-cystine is shown in the lower graph. Kinetic parameters were: V_{max} = 18.0 pmol/5 min/oocyte, K_m = 43 μ M and r (correlation coefficient) = 0.908, for the human rBAT (■); and V_{max} = 22.8 pmol/5 min/oocyte, K_m = 59 μ M and r = 0.897, for the rabbit rBAT (□).

[³⁵S]Methionine Labeling of Oocytes—Oocytes were injected with water or 35 ng of human rBAT cRNA in the presence or absence of 1.5 ng of tunicamycin in 50 nl of final volume. After 24 h, [³⁵S] methionine (0.5 μ Ci in 50 nl of water, ICN) was injected and the oocytes (usually a number of 10) were incubated for 30 h at 18 °C in 500 μ l of modified Barth's solution. The oocytes injected with tunicamycin were always incubated in the presence of 2 μ g/ml of this inhibitor. Oocytes were then harvested and kept in homogenization solution (0.25 M sucrose, 1 mM phenylmethylsulfonyl fluoride, and 1 μ g/ml leupeptin) at -20 °C until used.

Oocyte Membrane Purification and SDS-PAGE—Total oocyte membranes were purified as described elsewhere (33). Briefly, oocytes were homogenized by repeated passage through a 25-gauge needle in homogenization solution. The homogenate was pelleted (100,000 \times g) for 15 min at 4 °C and resuspended in 0.1 M Na₂CO₃ (7.5 ml). After 30 min on ice, samples were centrifuged (100,000 \times g) for 1 h at 4 °C and the final pellet was resuspended in homogenization solution (125 μ l/20 oocytes). Aliquots were used to quantify proteins according to Bradford (34) and to measure radioactivity content (cpm/ μ l) by liquid scintillation spectrometry. The volume corresponding to 50,000 cpm (approximately 8–12 μ g of oocyte membrane protein; *i.e.* equivalent to 1–2 of the starting oocytes) was mixed with 3 \times Laemmli sample buffer (35) and dithiothreitol to 100 mM was added. Prior to gel loading, samples were boiled for 5 min. The labeled proteins were separated by SDS-PAGE and visualized by autoradiography after enhancement with 1 M sodium salicylate (36).

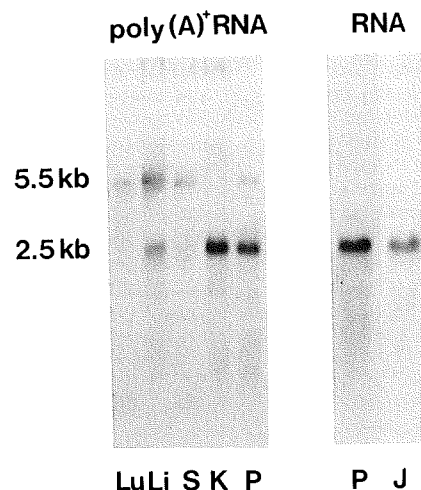


FIG. 4. Northern blot analysis for rBAT mRNA in human tissues. Human rBAT cDNA hybridizes to transcripts of ~2.5 and ~5.5 kb in length in poly(A)⁺ RNA from different human tissues. The short transcript is expressed predominantly in kidney (K), jejunum (J), and pancreas (P); a weaker signal for the lower transcript is also detectable in liver (Li). The long transcript is detected in poly(A)⁺ RNA from lung (Lu), skeletal muscle (S), liver (Li), and pancreas (P). For human poly(A)⁺ RNA (2 μ g), samples of a multiple tissue blot from Clontech was used. Total RNA (15 μ g) from jejunum and pancreas was loaded onto the gel and transferred to nylon. Blots were probed with ³²P-labeled human rBAT cDNA and washed at high stringency conditions (0.4 \times SSC, 0.1% SDS, 55 °C; see "Experimental Procedures"). Proper quality and/or transfer of RNA was substantiated by ethidium bromide staining (total RNA samples from jejunum and pancreas) or by hybridization with human β -actin cDNA (Clontech) use as a control probe (data not shown).

RESULTS AND DISCUSSION

A single clone (human rBAT) was isolated by screening a human kidney cortex cDNA library for expression of sodium-independent L-arginine uptake in *X. laevis* oocytes (data not shown). Injection of cRNA (2.5 ng/oocyte) synthesized from this clone increased the uptake of L-arginine (35.6 \pm 3.3 pmol/5 min/oocyte in cRNA-injected oocytes and 2.1 \pm 0.5 pmol/5 min/oocyte in water-injected oocytes; mean \pm S.E. from six independent experiments), L-leucine (25.2 \pm 3.0 pmol/5 min/oocyte in cRNA-injected oocytes and 1.7 \pm 0.5 pmol/5 min/oocyte in water-injected oocytes; n = 4) and L-cystine (15.2 \pm 1.9 pmol/5 min/oocyte in cRNA-injected oocytes and 0.35 \pm 0.05 pmol/5 min/oocyte in water-injected oocytes; n = 5). This induced amino acid transport activity resembles that elicited by the injection of rabbit rBAT and rat D2 cRNAs (1, 2). As shown in Fig. 1, the potency of the human rBAT cRNA-inducing transport of these amino acids in the oocytes is almost identical to that of rabbit rBAT cRNA.

To characterize all these induced uptake activities, we measured the mutual cis-inhibition by an excess concentration of the 3 amino acids tested. As shown in Fig. 2, A and B, L-arginine and L-leucine inhibited the induced uptake (*i.e.* calculated by subtracting the uptake values obtained in water-injected oocytes from the uptake values in cRNA-injected oocytes) of the 3 amino acids tested (*i.e.* L-cystine, L-arginine, and L-leucine). These inhibitions are almost complete at a 100-fold excess concentration (5 mM) of L-arginine and L-leucine (Fig. 2A). L-Cystine at 200 μ M, the highest reliable concentration in our incubation solutions, also inhibited the induced uptake of all the substrates tested (Fig. 2A). These results indicate saturability of the induced uptake of L-cystine, L-arginine, and L-leucine and are compatible with, but do not prove, the expression of a single component of transport. In

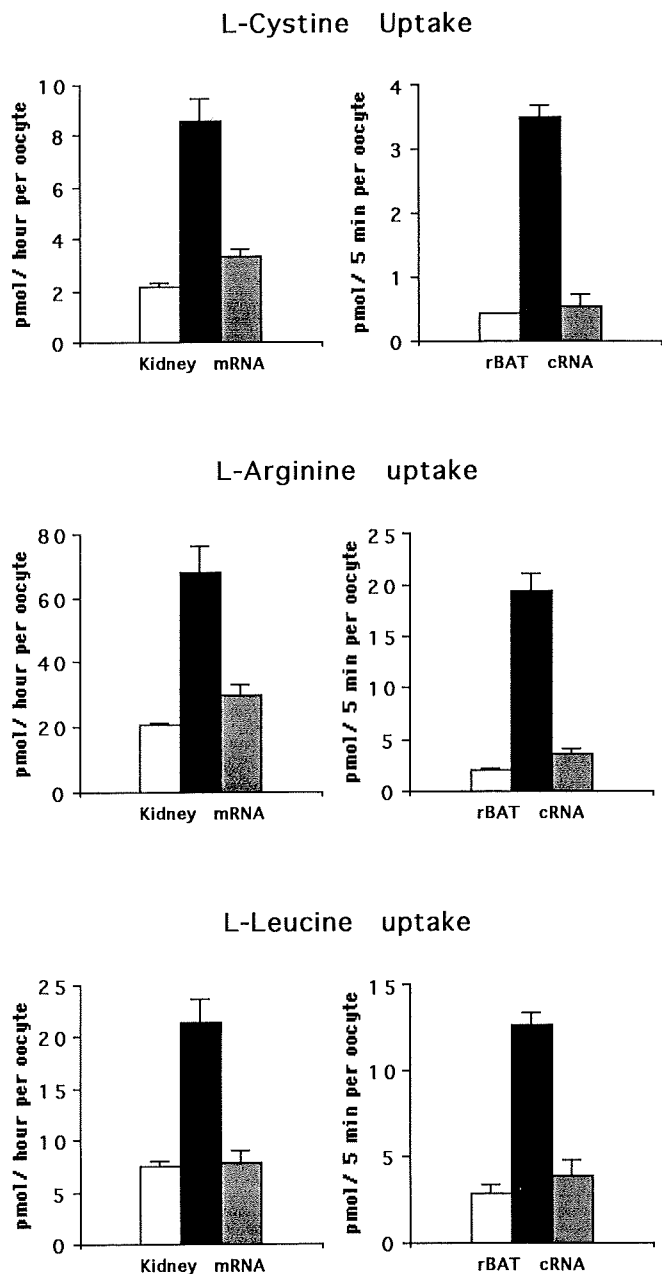


FIG. 5. Expression of amino acid uptake by human kidney poly(A)⁺ RNA in *Xenopus* oocytes; hybrid depletion with human rBAT antisense oligonucleotide. Oocytes were injected with water (open bars), 25 ng of human kidney poly(A)⁺ RNA (kidney mRNA) (solid and shaded bars), or 0.5 ng of human rBAT cRNA (solid and shaded bars). Prior to injection, mRNA (i.e. human kidney mRNA or rBAT cRNA) was incubated alone (solid bars) or in the presence (40 μ M) of a human rBAT antisense oligonucleotide (21-mer; see "Experimental Procedures") (shaded bars). Five (kidney mRNA) and 2 (rBAT cRNA) days after injection, uptake of 50 μ M L-cystine, L-arginine, and L-leucine was determined for 60-min (kidney mRNA) and 5-min (rBAT cRNA) incubations. Data are mean \pm S.E. from seven oocytes of a representative experiment.

keeping with this, rabbit rBAT cRNA induces transport of the above mentioned amino acids in the oocytes through a single component of transport (i.e. system b⁰⁺-like activity) (1). In addition to system b⁰⁺, different cell types (i.e. fibroblasts, cultured hepatocytes, and hepatoma cell lines) transport the anionic form of L-cystine through the plasma membrane via system X_c⁻, an antiporter for cystine and glutamic acid (37, 38). Interestingly, Groth and Rosenberg (39) dem-

onstrated that the renal transport defect seen in patients with cystinuria is not present in the cultured fibroblasts, suggesting that system X_c⁻ is not involved in cystinuria. The lack of inhibition of the human cRNA-induced L-cystine uptake by an excess of L-glutamic acid (Fig. 2A) preclude the expression of system X_c⁻ in our experiments.

The induced uptake of L-cystine (10–200 μ M) in the human and in the rabbit rBAT cRNA-injected oocytes showed complete saturation and an almost identical kinetic curve (Fig. 3). Indeed, both induced uptake activities fit well with a Michaelis-Menten kinetics (i.e. Eadie-Hofstee transformation; see Fig. 3) and with identical kinetic constants: apparent K_m in the micromolar range (i.e. 43 and 59 μ M for the human and rabbit rBAT, respectively) and V_{max} (i.e. 18 and 23 pmol/5 min/oocyte for the human and rabbit rBAT, respectively). These results fully agree with the expression of rBAT in *Xenopus* oocytes (1, 2). In contrast to the rBAT-induced uptake of L-cystine, the intrinsic uptake of this amino acid by water-injected (or uninjected) oocytes shows no saturability (Fig. 3), in keeping with previous reports (1, 3, 25).

Tissue expression of the mRNA corresponding to rBAT was examined by Northern blot analysis (Fig. 4). Human poly(A)⁺ RNA samples from different tissues showed mRNA species of ~2.5 and ~5.5 kb that hybridized with human rBAT cDNA. The short transcript is expressed predominantly in kidney, small intestine, and pancreas, and only a weak signal was detected in liver. In addition, a longer rBAT transcript (i.e. ~4 kb in length) is visible in total RNA from rabbit and rat kidney and small intestine (1, 2). The short and long rBAT mRNA species from rabbit and rat (i.e. rBAT-1 and rBAT-2) code essentially for the same protein but the latter shows an alternative polyadenylation and contains a UA-rich 3'-end (6). In contrast, the long transcript (i.e. ~5.5 kb in length) detected in human tissues most probably represents a wide distributed mRNA species with high homology to rBAT. With the exception of kidney, the long transcript is present in poly(A)⁺ RNA from all the tissues examined (i.e. those tissues shown in Fig. 4, and brain, placenta, and heart; data not shown). This long transcript was not detected when total RNA was used (i.e. in jejunum and pancreas; Fig. 4, or kidney and liver; data not shown). Similarly, rabbit and rat brain and rat heart show transcripts that hybridize with rBAT (or rat NAA-Tr) of ~5 kb in length (1, 5). It has been shown in rat tissues, by RNase protection assay, that these transcripts represent homologous NAA-Tr cross-reacting mRNA species (5).

To demonstrate further that the human rBAT cDNA isolated from the expression library is present in human kidney we searched for rBAT expression by human kidney poly(A)⁺ RNA in *Xenopus* oocytes. Injection of 25 ng (i.e. maximum dose) of human kidney poly(A)⁺ RNA resulted in the expression of L-cystine, L-arginine, and L-leucine uptake in the oocytes (Fig. 5). These results are compatible with the expression of system b⁰⁺ (1, 2, 30). To test further for a relationship of these induced uptake activities to rBAT-system b⁰⁺-like transport, we performed hybrid-depletion experiments of human kidney poly(A)⁺ RNA (25 ng/oocyte) with 40 μ M of an antisense oligonucleotide (to a coding region of human clone rBAT; see legend to Fig. 5 and "Experimental Procedures" for details). As shown in Fig. 5, rBAT hybrid depletion of human kidney mRNA (prior to oocyte injection) resulted in an almost complete block in mRNA-induced L-cystine, L-arginine, and L-leucine uptake. Indeed, the residual induced uptake in the experiment shown in Fig. 5 was 17% for L-cystine, 19% for L-arginine, and 3% for L-leucine. In the same experiment, hybrid depletion of human rBAT cRNA (0.5 ng/

-43	C CTT ACT GCA GGA AGG CAC TCC GAA GAC ATA AGT CGG TGA GAC	1201	TTT ATC CAA GAA GCT GAT TTT CCC TTC AAC AAT TAC CTC AGC ATG CTA GAC ACT GTT TCT
1	ATG GCT GAA GAT AAA AGC AAG AGA GAC TCC ATC GAG ATG AGT ATG AAG GGA TGC CAG ACA	401	Phe Ile Gln Glu Ala Asp Phe Pro Phe Asn Asn Tyr Leu Ser Met Leu Asp Thr Val Ser
1	Met Ala Glu Asp Lys Ser Lys Arg Asp Ser Ile Glu Met Ser Met Lys Gly Cys Gln Thr	1261	GGG AAC AGC GTG TAT GAG GTT ATC ACA TCC TGG ATG GAA AAC ATG CCA GAA GGA AAA TGG
61	AAC AAC GGG TTT GTC CAT AAT GAA GAC ATT CTG GAG CAG ACC CCG GAT CCA GGC AGC TCA	421	Gly Asn Ser Val Tyr Glu Val Ile Thr Ser Trp Met Glu Glu Asn Met Pro Glu Gly Lys Trp
21	Asn Asn Gly Phe Val His Asn Glu Ala Ile Leu Glu Gln Thr Pro Asp Tyr Trp Lys Ser	1321	CCT AAC TGG ATG ATT GGT GGA CCA GAC AGT TCA CGG CTG ACT TCG CGT TTG GGG AAT CAG
121	ACA GAC AAC CTG AAG CAC AGC ACC AGG GGC ATC CTT GGC TCC CAG GAC CCC GAC TTC AAG	441	Pro Asn Trp Met Ile Gly Gly Pro Asp Ser Ser Arg Leu Thr Ser Arg Leu Gly Asn Gln
41	Thr Asp Asn Leu Lys His Ser Thr Arg Gly Ile Leu Gly Ser Gln Glu Pro Asp Phe Lys	1381	TAT GTC AAC GTG ATG AAC ATG CTT CTT TTC ACA CTC CCT GGA ACT CCT ATA ACT TAC TAT
181	GGC GTC CAG CCC TAT GCG GGG ATG CCC AAG GAG GTG CTG TTC CAG TTC TCT GGC CAG GCC	461	Tyr Val Asn Val Met Asn Met Leu Leu Phe Thr Leu Pro Gly Thr Pro Ile Thr Tyr Tyr
61	Gly Val Gln Pro Tyr Ala Glu Gly Met Pro Lys Glu Val Leu Phe Gln Phe Ser Gly Gln Ala	1441	GGA GAA GAA ATT GGA ATG GGA AAT ATT GTA GCC GCA AAT CTC AAT GAA AGC TAT GAT ATT
241	CGC TAC CGC ATA CCT CGG GAG ATC CTC TTC TGG CTC ACA GTG GCT TCT GTG CTG GTG CTC	481	Gly Glu Glu Ile Gly Met Gly Asn Ile Val Ala Ala Asn Leu <u>Asn Glu Ser</u> Tyr Asp Ile
81	Arg Tyr Arg Ile Pro Arg Glu Ile Leu Phe Trp Leu Thr Val Ala Ser Val Leu Val Leu	1501	AAT ACC CTT CGC TCA AAG TCA CCA ATG CAG TGG GAC AAT AGT TCA AAT GCT GGT TTT TCT
301	ATC GCG GCC ACC ATA GCC ATC ATT GCC CTC TCT CCA AAG TGC TCA GAC TGG TGG CAG GAG	501	Asn Thr Leu Arg Ser Lys Ser Pro Met Lys Trp Asp <u>Asn Ser Ser</u> Asn Ala Gly Phe Ser
101	Arg Glu Val Asp Pro Ile Phe Gly Thr Met Glu Asp Phe Glu Asn Leu Tyr Trp Lys Gln Glu	1561	GAA GCT AGT AAC ACC TGG TTA CCT ACC AAT TCA GAT TAC CAC ACT GTG AAT GTT GAT GTC
361	GGG CCC ATG TAC CAG ATC TAC CCA AGG TCT TTC AAG GAC AGT AAC AAG GAT GGG AAC GGA	521	Glu Ala Ser Asn Thr Trp Leu Pro Thr Asn Ser Asp Tyr His Thr Val Asn Val Asp Val
121	Gly Pro Met Tyr Gln Ile Tyr Pro Arg Ser Phe Lys Asp Ser Asn Lys Asp Gly Asn Gly	1621	CAA AAG ACT CAG CCC AGA TCG GCT TTG AAG TTA TAT CAA GAT TTA AGT CTA CTT CAT GCC
421	GAT CTG AAA GGT ATT CAA GAT AAA CTG GAC TAC ATC ACA GCT TTA AAT ATA AAA ACT GTT	541	Gln Lys Thr Pro Arg Ser Thr Arg Ser Pro Lys Thr Arg Ser Leu Ser Leu Leu His Ala
141	Asp Leu Val Ser Pro Ile Phe Gly Thr Met Glu Asp Phe Thr Ala Leu Val Ala Ala Ile	1681	AAT GAG CTA CTC CTC AAC AGG GGC TGG TTT TGC CAT TTG AGG AAT GAC AGC CAC TAT GTT
481	TGG ATT ACT TCA TTT TAT AAA TCG TCC CTT AAA GAT TTC AGA TAT GGT GTT GAA GAT TTC	561	Asn Glu Leu Leu Leu Asn Arg Gly Trp Phe Cys His Leu Arg <u>Asn Asp Ser</u> His Tyr Val
161	Trp Ile Thr Ser Phe Tyr Lys Ser Ser Leu Lys Asp Phe Arg Tyr Gly Val Glu Asp Phe	1741	GTG TAC ACA AGA GAG CTG GAT GGC ATC GAC AGA ATC TTT ATC GTG GTT CTG AAT TTT GGA
541	CGG GAA GTT GAT CCC ATT TTT GGA ACG ATG GAA GAT TTT GAG AAT CTG GTT GCA GCC ATA	581	Val Tyr Thr Arg Glu Leu Asp Gly Ile Asp Gly Thr Ser Leu Val Val Leu Asn Phe Leu
181	Arg Thr Phe Gln Pro Ile Phe Gly Thr Arg Thr Gly Lys Tyr Thr Asp Tyr Thr Ile Trp His	1801	GAA TCA ACA CTG TTA AAT CTA CAT AAT ATG ATT TCG GGC CTT CCC GCT AAA ATG AGA ATA
601	CAT GAT AAA GGT TTA AAA TTA ATC ATC GAT TTC ATA CCA AAC CAC AGC AGT GAT AAA CAT	601	Glu Ser Thr Leu Leu Asn Leu His Asn Met Ile Ser Gly Leu Pro Ala Lys Met Arg Ile
201	His Asp Lys Gly Leu Lys Leu Ile Ile Asp Phe Ile Pro <u>Asn His Thr</u> Ser Asp Lys His	1861	AGG TTA AGT ACC AAT TCT GCC AAC AAA GGC AGT AAA GTT GAT ACA AGT GGC ATT TTT CTG
661	ATT TGG TTT CAA TTG AGT CGG ACA CGG ACA GGA AAA TAT ACT GAT TAT TAT ATC TGG CAT	621	Arg Leu Ser Thr Asn Ser Thr Asn Ser Ala Asp Lys Thr Arg Ser Leu Ser Leu Leu His Ala
221	Ile Trp Phe Gln Thr His Phe Ser Glu Val Arg Asn Gln Cys Tyr Phe His Gln Phe Met Lys	1921	GAC AAG GGA GAG GGA CTC ATC TTT GAA CAC AAC ACG AAG AAT CTC CTT CAT CGC CAA ACA
721	GAC TGT ACC CAT GAA AAT GGC AAA ACC ATT CCA CCC AAC AAC TGG TTA AGT GTG TAT GGA	641	Asp Lys Gly Glu Gly Leu Ile Phe Glu His Asn Thr Lys Asn Leu Leu His Arg Gln Thr
241	Asp Cys Thr His Glu Asn Gly Lys Thr Ile Pro Pro Asn Asn Trp Leu Ser Val Tyr Gly	1981	GCT TTC AGA GAT AGA TGC TTT GTT TCC AAT CGA GCA TGC TAT TCC AGT GTA CTG AAC ATA
781	AAC TCC AGT TGG CAC TTT GAC GAA GTG CGA AAC CAA TGT TAT TTT CAT CAG TTT ATG AAA	661	Ala Phe Arg Asp Arg Cys Phe Val Ser Asn Lys Leu Cys Tyr Ser Ser Val Leu Asn Ile
261	<u>Asn Ser Ser</u> Trp His Phe Ser Glu Val Arg Asn Gln Cys Tyr Phe His Gln Phe Met Lys	2041	CTG TAT ACC TCG TGT TAG GCACCTTTATGAAGAGATGAAGACACTGGCATTTCAGTGGGATTGTAAGCATTTG
841	GAG CAA CCT GAT TTA AAT TTC CGC AAT CCT GAT GTT CAA GAA ATA AAA GAA ATT TTA	681	Leu Tyr Thr Ser Cys ***
281	Glu Gln Pro Asp Leu Asn Phe Arg Asn Pro Asp Val Gln Glu Glu Ile Lys Glu Ile Leu	2114	TAATAGCTTCATGACAGCATGCTCTGGTGGAAACAATCATTAAATCTTCGATATTTCTGTAGCTTGAATGTAAGTCT
901	CGG TTC TGG CTC ACA AAG GGT GTT GAT GGT TTT AGT TTG GAT GCT GTT AAA TTC CTC CTA	2193	TTAAGAAAGGTTCTCAATGTTTTGAAAAAATAAAATGTTTAAAAGTAAAAAATAAAAAAATAAAAAA
301	Arg Phe Trp Leu Thr Lys Gly Val Asp Gly Phe Ser Leu Asp Ala Val Lys Leu Leu		
961	GAA GCA AAG CAC CTG AGA GAT GAG ATC CAA GTA AAT AAG ACC CAA ATC CCG GAC ACG GTC		
321	Glu Ala Lys His Leu Arg Asp Glu Ile Gln Val <u>Asn Lys Thr</u> Gln Ile Pro Asp Thr Val		
1021	ACA CAA TAC TCG GAG CTG TAC CAT GAC TTC ACC ACC ACG CAG GTG GGA ATG CAC GAC ATT		
341	Thr Gln Tyr Ser Glu Leu Tyr His Asp Phe Thr Thr Thr Gln Val Gly Met His Asp Ile		
1081	GTC CGC AGC TTC CGG CAG ACC ATG GAC CAA TAC AGC ACG GAG CCC GGC AGA TAC AGG TTC		
361	Val Arg Ser Phe Arg Gln Thr Met Asp Gln Tyr Ser Thr Glu Pro Gly Arg Tyr Arg Phe		
1141	ATG GGG ACT GAA GCC TAT GCA GAG AGT ATT GAC AGG ACC GTG ATG TAC TAT GGA TTG CCA		
381	Met Gly Thr Glu Ala Tyr Ala Glu Ser Ile Asp Arg Thr Val Met Tyr Tyr Gly Leu Pro		

FIG. 6. Nucleotide and deduced amino acid sequence of clone human rBAT. The first line shows the nucleotide sequence of rBAT cDNA. Nucleotides are numbered in the 5' to 3' direction, starting with the first nucleotide of the first ATG codon. The deduced amino acid sequence is shown below. The size of the transcript is 2304 base pairs and contains a poly(A) tail of 21 base pairs. The first ATG codon in the described open reading frame lies within a consensus initiation sequence (39). The stop codon (TAG) is indicated by three stars. The possible polyadenylation signal (AATAAA) is underlined. Six potential N-glycosylation sites (Asn-Xaa-Ser or Asn-Xaa-Thr) are underlined.

oocyte) with human rBAT antisense oligonucleotide (40 μ M) blocks almost completely cRNA-induced amino acid uptake (i.e. residual induced uptake of 4, 9, and 9% for L-cystine, L-arginine, and L-leucine uptake, respectively). These results indicate that rBAT-related transcripts are responsible for most, if not all, human kidney mRNA-induced uptake of these amino acids in oocytes; the residual kidney mRNA-induced uptake of L-cystine and L-arginine after rBAT-hybrid depletion, if it is significant, is in any case too small to be analyzed. In keeping with this, kidney mRNA-induced uptake of L-arginine and L-leucine is greatly impaired by an excess concentration in the uptake media of L-leucine and L-arginine, respectively (i.e. kidney mRNA-induced uptake of 50 μ M L-arginine (47.2 ± 8.3 pmol/h/oocyte) is inhibited >70% by 5 mM L-leucine (14.0 ± 2.6 pmol/h/oocyte) and kidney mRNA-induced uptake of 50 μ M L-leucine (13.9 ± 2.3 pmol/h/oocyte) is inhibited >95% by 5 mM L-arginine (0.3 ± 0.9 pmol/h/oocyte)). Similarly, human rBAT cRNA-induced amino acid uptake is greatly impaired by an excess concentration in the uptake media of L-leucine and L-arginine (i.e. inhibition of 85 and 71% for L-arginine uptake (50 μ M) in the presence of L-leucine (5 mM) and inhibition of 96 and 90% for L-leucine uptake (50 μ M) in the presence of L-arginine (5 mM) for the two independent experiments shown in Fig. 2). Similarly, an almost complete rBAT-hybrid depletion was obtained with

rat and rabbit kidney mRNA: rBAT-hybrid-depletion resulted in inhibitions of >95% for L-cystine and ~85% for L-arginine uptake induced by rat kidney mRNA and in inhibitions of ~85% for L-arginine and of ~95% for L-leucine uptake induced by rabbit kidney mRNA (data not shown). In contrast to these rBAT-hybrid depletion experiments with human, rat, and rabbit kidney mRNA, rBAT-hybrid depletion of rabbit jejunum mRNA resulted in the abolition of L-cystine uptake induction and in partial L-arginine and L-leucine uptake induction (3). This indicates that rabbit jejunum mRNA expresses significantly in oocytes other sodium-independent amino acid transport systems for L-arginine (i.e. system y^+ /murine leukemia virus receptor (26), system y^+ -like/antigen 4F2hc (28), and/or an unknown amino acid transport related mRNA) and for L-leucine (i.e. probably an L-type system). In conclusion, human kidney poly(A)⁺ RNA, similarly to rat and rabbit kidney mRNA, shows functional rBAT-related transcript(s) responsible for most, if not all, sodium-independent transport activity of L-cystine, L-arginine, and L-leucine expressed in *Xenopus* oocytes.

The nucleotide and deduced amino acid sequences of human rBAT are shown in Fig. 6. The size of the rBAT clone (2304 base pairs) corresponds fairly well to that of the transcript seen on Northern blots of kidney and jejunum RNA. The first ATG codon lies within a good consensus initiation sequence

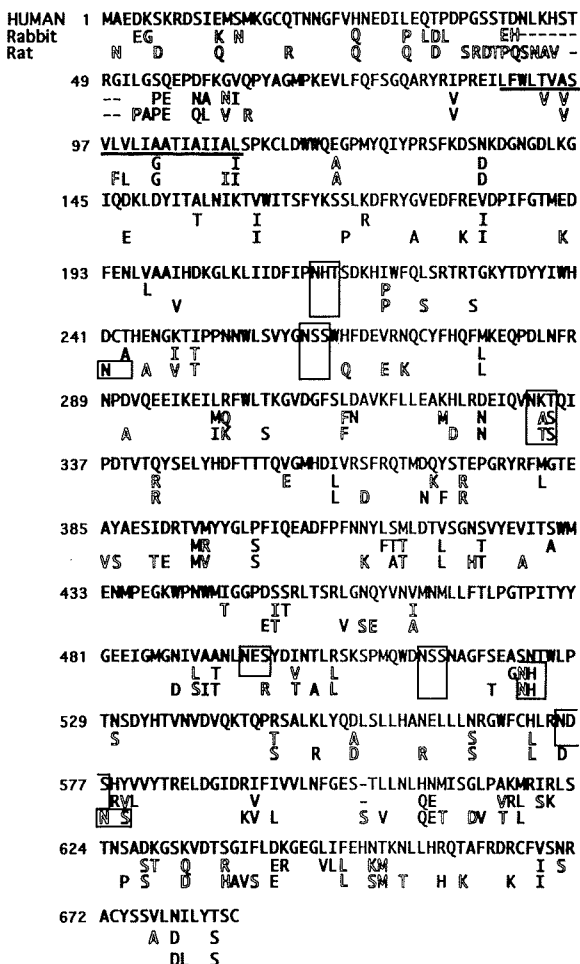


FIG. 7. Comparison of the predicted amino acid sequences of human, rabbit, and rat rBAT proteins. The first line shows the human rBAT amino acid sequence. Below, only substituted amino acids in the sequences of rabbit (second line) and rat (third line) rBAT proteins are shown. Conserved amino acid substitutions are indicated by normal text, whereas nonconservative substitutions, in at least one of the three sequences, are outlined in the rabbit and rat rBAT proteins. Amino acid gaps in the sequences are indicated by dashes; the human rBAT protein is 685 amino acid residues long, whereas rabbit and rat rBAT proteins are 677 and 683 amino acids long, respectively. The human rBAT protein, in this alignment, is 85% identical to the rabbit rBAT (93% similarity) and 80% to the rat rBAT (89% similarity). The putative transmembrane domain, as deduced by hydrophobicity analysis (40), is heavily underlined only in the human protein. Six (human protein) and seven (rabbit and rat proteins) potential *N*-glycosylation sites are boxed.

(i.e. a purine (A) in position -3 and a G in position +4; Ref. 39). The open reading frame continues to the first stop codon (TAG) at base 2056 and codes for 685 amino acid residues with molecular mass of 78,852 Da. As expected from the transport activity induced by the isolated human cDNA in oocytes, the human rBAT protein shows a high degree of homology with the rabbit (85% identity with rBAT; Ref. 1) and rat (80% identity with NAA-Tr or D2; Refs. 2 and 4) deduced rBAT proteins (Fig. 7). Accordingly, the three rBAT cDNA clones also show a high level of homology (i.e. 77 and 83% identity of the rat (NAA-Tr or D2) and rabbit (rBAT) cDNAs, respectively, with the human rBAT cDNA). Amino acid residue substitutions are significantly conserved among the three deduced rBAT proteins (i.e. 93 and 89% similarity of human rBAT protein with the rabbit and rat deduced proteins, respectively). As a consequence of the high level of homology, the three deduced proteins show five identical out

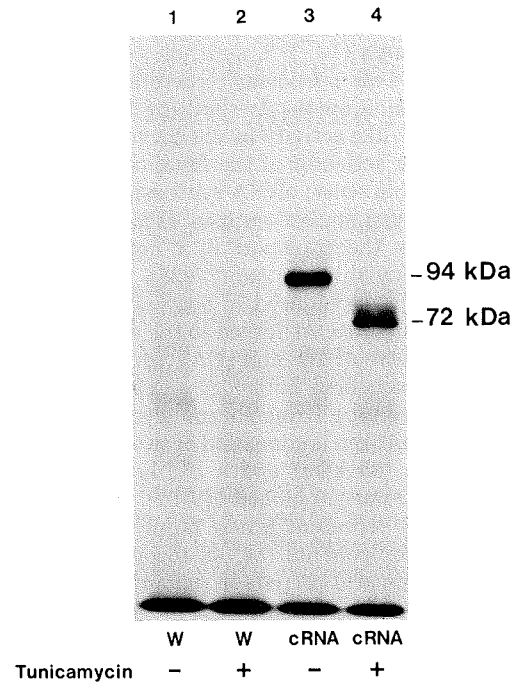


FIG. 8. Synthesis of human rBAT protein by *Xenopus* oocytes. Oocytes were injected with 50 nl of water (lanes 1 and 2) or with 50 nl of water containing 35 ng of human rBAT cRNA (lanes 3 and 4). Tunicamycin (1.5 ng/oocyte) was co-injected with water or rBAT cRNA (lanes 2 and 4). Oocytes injected with tunicamycin were kept, for the rest of the experiment, in Barth's solution also containing the *N*-glycosylation inhibitor (2 μ g/ml). Twenty-four hours later, oocytes were injected with [35 S]methionine (0.5 μ Ci/oocyte) (lanes 1-4). Thirty hours after [35 S]methionine injections, the oocytes (20 per each condition) were placed into homogenization buffer and stored at -20 $^{\circ}$ C for total membranes extraction (see "Experimental Procedures"). Samples of membrane proteins containing an identical amount of 35 S radioactivity (50,000 cpm; corresponding to 8-12 μ g of protein) were loaded onto a 7.5% polyacrylamide gel for SDS-PAGE, and fluorography was developed after 16 h of exposure to photographic film (see "Experimental Procedures").

of a total of seven potential *N*-glycosylation sites and an identical hydrophobicity plot (40) (data not shown) which suggests the existence of a single transmembrane domain (residues 89-110 in the human rBAT protein; Fig. 7). In fact, previous reports suggested that rBAT (or D2 in the rat) protein appears to be a type II membrane glycoprotein (1, 2). Tate and co-workers (4) suggested that NAA-Tr deduced protein (i.e. rat rBAT) contains, in addition to the above mentioned transmembrane domain, three other putative membrane-spanning domains. Experimental evidence is needed to elucidate the mechanism of rBAT insertion into cell membranes.

Translation products of cRNA injected into oocytes could be revealed by [35 S]methionine labeling (41). As shown in Fig. 8, *Xenopus* oocyte translation of human rBAT cRNA resulted in a cRNA-specific protein band of ~94 kDa in crude oocyte membranes. Treatment of the human rBAT cRNA-injected oocytes with tunicamycin shifts the translation product to a lower molecular mass (~72 kDa). The size of this band fits reasonably well with the size of the protein deduced from the predicted open reading frame. These observations indicate that human rBAT cDNA codes for an *N*-glycosylated membrane protein.

Known plasma membrane carriers for organic and inorganic substrates in mammals (e.g. Refs. 30, 32, 42-48) have, as a common structural feature, a variable number (i.e. 6-12) of putative membrane-spanning domains. In contrast, rBAT-

deduced proteins contain a lower number of putative transmembrane segments (*i.e.* 1–4, depending on structure prognosis) (1, 2, 4). This particular structural feature of rBAT prompted discussion on the mechanism by which rBAT induces system b⁰⁺-like activity in the oocytes: rBAT might be a monomer or an activator of the functional b⁰⁺ amino acid transporter. In this sense, cRNA synthesized from the heavy chain of the human surface antigen 4F2 cDNA (4F2hc) induces in oocytes the transport of L-arginine and Na⁺/L-leucine through a y⁺-like system (27–28). In addition, part of the L-arginine uptake induced in oocytes by rabbit jejunum poly(A)⁺ RNA might be attributed to the expression of 4F2hc messenger RNA (3). 4F2hc and rBAT proteins show significant amino acid sequence homology and a similar hydrophobicity, as putative type II membrane glycoproteins (27). Interestingly, 4F2 antigen is composed of two different subunits, the heavy chain (85 kDa) and a highly hydrophobic light subunit (40 kDa) not yet cloned (49–52). This suggests that rBAT and 4F2hc represent a new family of proteins which act as specific components (*i.e.* as modulator or supporter subunits) of amino acid carriers in mammals.

In conclusion, we have isolated a functional cDNA clone corresponding to the human rBAT. Messenger RNA for rBAT is present predominately in human kidney, jejunum, and pancreas. In addition, rBAT mRNA from human kidney expresses uptake of L-cystine, L-arginine, and L-leucine in oocytes. The induced uptake of L-cystine is mostly, if not completely, due to the expression of rBAT mRNA. rBAT cRNA induces in oocytes transport of cystine through a b⁰⁺-like system that shows high affinity for L-dibasic amino acids (1). In contrast, the 4F2hc surface antigen and the murine leukemia virus receptor, the two other known proteins related to dibasic amino acid transport present in renal and intestinal tissues, do not induce cystine transport (27, 28). Human cystinurias are genetic diseases that involve abnormalities of intestinal and renal absorption of cystine and dibasic amino acids, which lead to malabsorption and hyperexcretion of these amino acids with normal plasma levels (11, 12). A defect in brush-border membrane b⁰⁺ activity might be involved in cystinuria. Substrate specificity of rBAT-induced uptake and tissue distribution of rBAT mRNA is consistent with this. In this case, intact transport systems that carry the neutral amino acids transported by system b⁰⁺ (*e.g.* neutral brush-border (7)) would compensate for the absorption of these amino acids. The hypothesis that human rBAT might be involved in human cystinuria is currently under study.

Acknowledgments—We thank Dr. F. Rousaud, P. Real, D. Haro, B. Lloberas, and A. Fabra for samples of human tissues and RNA. We are grateful to A. Bosch, M. Chillón, and J. M. Soria for assistance during the sequencing of the cDNA clone, and to A. Gaona and M. Miranda for synthesis of oligonucleotides. We thank Robin Rycroft for his editorial help.

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