The rBAT Gene Is Responsible for L-Cystine Uptake via the b⁰⁺⁺-like Amino Acid Transport System in a “Renal Proximal Tubular” Cell Line (OK Cells)*

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Several studies have shown that the cRNA of human, rabbit, or rat rBAT induces in Xenopus oocytes sodium-independent, high affinity uptake of L-cystine via a system b⁰⁺⁺-like amino acid exchanger. We have shown that mutations in rBAT cause type I cystinuria (Calonge, M. J., Gasparini, P., Chillarón, J., Chillón, M., Gallucci, M., Rousaud, F., Zelante, L., Testar, X., Dallapiccola, B., Di Silverio, F., Barceló, P., Estivill, X., Zorzano, A., Nunes, V., and Palacín, M. (1994) Nat. Genet. 6, 420–425; Calonge, M. J., Vololini, V., Biscaglia, L., Rousaud, F., De San tis, L., Beccia, E., Zelante, L., Testar, X., Zorzano, A., Estivill, X., Gasparini, P., Nunes, V., and Palacín, M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9667–9671). Apart from oocytes, no other expression system has been used for transfection of functional rBAT activity. Furthermore, the b⁰⁺⁺-like transport activity has not been clearly described in the kidney or intestine. Here, we report that a “proximal tubular-like” cell line derived from opossum kidney (OK cells) expresses an rBAT transcript. Poly(A)⁺ RNA from OK cells induced system b⁰⁺⁺-like transport activity in oocytes. This was hybrid-depleted by human rBAT antisense oligonucleotides. A polymerase chain reaction-amplified cDNA fragment (≈700 base pairs) from OK cell RNA corresponds to an rBAT protein fragment 65–69% identical to those from human, rabbit and rat kidneys. We have also examined transport of L-cystine in OK cells and found characteristics very similar to the amino acid exchanger activity induced by rBAT cRNA in oocytes. Uptake of L-cystine was of high affinity, sodium-independent and shared with L-arginine and L-leucine. This was trans-stimulated by amino acids with the same specificity as rBAT-induced transport activity in oocytes. Furthermore, it was localized to the apical pole of confluent OK cells. To demonstrate that the rBAT protein is functionally related to this transport activity, we have transfected OK cells with human rBAT antisense and sense sequences. Transfection with rBAT antisense, but not with rBAT sense, resulted in the specific reduction of rBAT mRNA expression and b⁰⁺⁺-like transport activity. These results demonstrate that rBAT is functionally related to the L-cystine uptake via system b⁰⁺⁺-like in the apical pole of the renal OK cell line.

Human rBAT cDNA elicits high affinity sodium-independent transport of cystine, dibasic amino acids, and some zwitterionic amino acids via a b⁰⁺⁺-like transport system in Xenopus oocytes (1, 2). Heteroexchange diffusion of these amino acids has been reported for this transport activity expressed by rabbit (3, 4), rat (5), and human rBAT cRNA in oocytes. rBAT protein is expressed in the brush border plasma membrane of both the proximal straight tubules of the nephron and the small intestine (6, 7). Recent studies have demonstrated that mutations in the human rBAT gene cause cystinuria (8–12). Cystinuria is a common inherited aminoaciduria due to the defective transport of cystine and dibasic amino acids through the epithelial cells of the renal tubule and intestinal tract (13, 14). The clinical manifestation of cystinuria is the development of kidney cystine calculi resulting from the poor solubility of this amino acid (14). Three types of classic cystinuria have been described based on the basis of the amino acid hyperexcretion of heterozygotes and the degree of the intestinal transport defect (14–16). Very recently, strong evidence has been offered suggesting that rBAT is only responsible for type I (10, 12).

Due to the role of the rBAT gene in type I cystinuria, the rBAT protein is considered to be responsible for the reabsorption of cystine and dibasic amino acids in the proximal straight tubule. Two aspects of this remain to be clarified. First, a complete picture of the b⁰⁺⁺-like transport activity responsible for cystine reabsorption has not been described in the epithelial cells of kidney or intestine. In renal brush border membrane vesicles a high affinity system for cystine and dibasic amino acids, which shows heteroexchange diffusion between these substrates, has been demonstrated (17–19). This transport was shown to be defective in biopsies of intestinal mucosa from cystinuric patients (20–22). The sodium dependence of this reabsorption system is controversial, as is its interaction with neutral amino acids, since no hyperexcretion of neutral amino acids occurs in cystinuria (17–19, 22–24). Second, the role of rBAT in the b⁰⁺⁺-like transport mechanism is unknown.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank† EMBL Data Bank with accession number(s) X95475.

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dence from Tate and co-workers (25) suggests a four-membrane-spanning domain model for rBAT. This topology is unusual for metabolite transporters, which appear to contain 8–12 transmembrane domains (26). This fostered the idea that rBAT is a regulatory subunit of an oligomeric transporter rather than the transporter itself (27, 28). Indirect evidence suggested that rBAT forms a heterodimeric structure of 125 kDa with an unidentified protein of 40–50 kDa in renal brush border membranes and oocytes.2 In addition, transient expression of rBAT in COS cells revealed either expression of rBAT in the cell surface without concomitant amino acid transport activity (30) or a protein product that does not reach the plasma membrane.3 Thus, no cell system other than oocytes has shown expression of amino acid transport activity associated with rBAT. It thus remains an open question whether the amino acid transport activity associated with rBAT in the renal epithelial cells is the same as that elicited by rBAT expression in oocytes.

To this end we searched for the role of rBAT in the transport of cystine in an epithelial cell line derived from proximal tubules of the kidney of an American opossum, the cell line OK. These cells express an mRNA transcript that hybridizes with rBAT probes (27, 28), and they show high affinity sodium-independent cystine-dibasic amino acid transport in the apical pole (31). Here we demonstrate that OK cells express rBAT mRNA and that the transport of cystine in the apical pole of these cells is due to an amino acid transport system very similar to the b0,+l-like system elicited by rBAT in oocytes. Transfection of rBAT antisense sequences results in the specific reduction of rBAT mRNA expression and b0,+l-like transport activity. This demonstrates that rBAT is responsible for the high affinity sodium-independent l-cystine uptake shared with dibasic and neutral amino acids (i.e. system b0,+l) present in the apical plote of the renal cell line OK.

MATERIALS AND METHODS

Cell Culture and Uptake Studies in OK Cells—OK cells were purchased with the OK cell line clone 3B2, derived by selection from the original OK cells (32), between passages 16 and 21. This clone was originally obtained from Dr. A. Gaby, Geneva, Switzerland, with 50 nmoi of poly(A)+ RNA. The OK cells are healthy looking stage VI oocytes were injected with OK cell RNA at 1–3 × 10⁶ cells/ml. OK cells were defolliculated by collagenase D (Boehringer Mannheim) and then washed in MGA medium. Uptake was initiated by the addition of 500 μl of the uptake medium to the basolateral side of the cell monolayer.

For transport studies, OK cells were grown on Handmade transwell filters (Nucleopore Corp. filtration products). A 1-cm diameter polycarbonate tube was cut in 1-cm sections, and polycarbonate filters (0.2-μm pore size, 12-cm diameter, Nuclepore) were glued with 1:1 cyclohexanone/ chloroform to one side of each section. The filters were sterilized, cleaned and incubated at 37°C for at least 1 h, dried overnight, and collagen-coated with 50 μl (0.5 μl/filter) of rat tail collagen (R-type, Serva; 5 mg/ml in 50% ethanole). Cells were seeded at high density 1.3 from the final trypsinized volume (20 ml of cell suspension). 50 μl of MGA medium was added to the basal surface. Cells were refed 1 day before the uptake, which was carried out 3 days after the seeding, when cells had reached confluence. Transport measurements were performed using the uptake medium described above for plastic cell dishes. Both sides of the plastic transwell filters were thoroughly washed in MGA medium. For apical transport, in either the presence or absence of sodium, the apical side was immersed in MGA medium and stimulated by the addition of 500 μl of the uptake medium to the corresponding side of the filter. For basal transport, the same procedure was followed, i.e. the apical side was immersed in MGA medium. Uptake was stopped by aspirating the uptake solution and washing the filters five times in the stop solution mentioned above. 100 μl of 0.5% Triton X-100 was added to each filter, and, 30 min later, total radioactivity incorporated into the monolayer was measured by liquid scintillation counting of the whole filter immersed in 9 ml of scintillation fluid. Results are expressed as pmol/30 μl.

Oocyte Studies—Oocyte management, injection, and amino acid uptake measurements were described elsewhere (1). Xenopus laevis (H. Kühler, Institut für Entwicklungsbiologie, Hamburg, Germany) oocytes were dejuked by digestion with collagenase D (Boehringer Mannheim) and healthy-looking stage VI oocytes were injected (Inject-matic system, J. A. Gabay, Geneva, Switzerland) with 50 nl of poly(A)+ RNA, or water, as described elsewhere (1). RNA was extracted from OK cells by the guanidinium thiocyanate-phenol-chloroform method as described (36). Oligo(T)-cellulose (Boehringer Mannheim) for the purification of poly(A)+ RNA was used following the manufacturer’s protocol.

For hybrid depletions experiments, OK cell mRNA (0.7–1.0 mg/ml) or human rBAT cRNA (0.01 mg/ml), prepared as described elsewhere (1), were denatured at 65°C for 5 min in a solution containing 50 μm NaCl and 20 μM of a 20-mer sense or a 21-mer antisense oligonucleotide complementary to the human (and rat) rBAT mRNA sequence (sense primer: 5'-TGG AAT AGG TAC TCA TCT AAG TGC-3'; antisense: 5'-GAA CAG CAC CTC CCT GGG CAT-3'; GAPDH; TATACTACAAGCTTTCAGCGTTG; 5'-CCA AGC GAC TCC CTG CCT CAT-3'; at base 222 of the coding region; antisense: 5'-GAA CAG CAC CTC CCT GGG CAT-3'; at base 222 of the coding region) and further incubated at 42°C for 30 min prior to oocyte injection. In these conditions, human rBAT cRNA-induced amino acid transport activity was specifically depleted by >80% by the above mentioned antisense oligonucleotide (data not shown).

PCR Amplification and DNA Sequencing—cDNA was synthesized in a 20-μl reaction volume from a 1-μl RNA aliquot (poly(A)+ RNA from OK cells at 1–3 μg/ml) and primed with 2.5 μM oligo(DT)16 (GeneAmp RNA PCR kit; Perkin-Elmer). Incubation was for 60 min at 42°C. Reverse transcriptase was inactivated at 99°C for 5 min. The 20-mer oligonucleotides C40 (sense) and CSR (antisense), complementary to the human rBAT cDNA sequence, elsewhere described (8), were used as primers for the PCR amplification that comprised: (a) an initial denaturation at 95°C for 3 min; (b) 5 cycles with denaturation at 95°C
for 35 s, annealing at 46 °C for 40 s, and extension at 74 °C for 70 s (after annealing, temperature increased at a ramp rate of 12 °C/min); and (c) 29 cycles with denaturation at 94 °C for 25 s, annealing at 56 °C for 25 s, and extension at 74 °C for 70 s. The reaction buffer was Perkin-Elmer Roche N808-0006 with 200 μM each dNTP, 0.6 μM of each primer, 2.5 mM MgCl₂, 10 μl of the reverse transcription reaction, and 2 μl of the PCR product (Perkin-Elmer Roche) in 100 μl. This amplification yielded a fragment of ~700 bp as expected. The PCR product was run in a 4% nondenaturing polyacrylamide gel, and the 700-bp band was cut and eluted with 20 μl of distilled water. In order to obtain large amounts of this fragment, 5 μl of the eluted product was used as a template in a second round of PCR with the same primers and at high stringency conditions for 34 cycles. After purification with QiaQuick columns (Qiagen DyeEx 2.0™ terminator cycle sequencing kit) this product was used for automatic DNA sequencing and as a DNA probe for Northern analysis.

The purified C4D-CSR PCR-Fragment was sequenced with the same primers as for amplification and the internal primers C4R and C5D, described elsewhere (8), using an automatic DNA sequencer (Applied Biosystems model 373A) and Taq DyeDeoxy™ terminator cycle sequencing kit.

Permanent Transfection of OK Cells—A 669-bp EcoRI/ClaI (Klenow blunt-ended) fragment from the full-length human rBAT cDNA (1), comprising 43 bp of the 5′ noncoding region, the ATG translation initiation codon and 626 bp of the coding sequence (including two bases of the codon for the amino acid residue Ile-209), was inserted in pXpDNA3 mammalian expression vector (Invitrogen Corp.) polynucleotides (linker previously cut with (a) BamHI (Klenow blunt-ended)/EcoRI for antisense orientation or (b) XhoI (Klenow blunt-ended)/EcoRI for sense orientation. The expression of both constructs was under the control of the constitutive cytomegalovirus promoter. After transformation, antisense construct clones and sense construct clones were isolated and constructions were checked by restriction analysis.

For transfections and clone selection, confluent OK cell monolayers (day 0) were trypsinized and seeded 1/2 in 25-cm² flasks. On day 1 (40–50% of confluence) cells were washed three times and then covered with 3 ml of serum-free medium. Cells were transfected by adding dropwise 120 μl of DNA-Lipofectin mixture to each flask and swirling gently. DNA-Lipofectin mixture (1:1, v/v) was prepared with human rBAT sense or antisense constructs (45 μg/60 μl) and Lipofectin (30 μg/60 μl), following supplier’s protocol (Life Technologies, Inc.). Cells were incubated with DNA-Lipofectin-containing medium for 16 h in a humidified atmosphere of 5% CO₂, 95% air at 37°C. Medium was then removed and replaced by complete medium (i.e. with 10% serum). Cells were grown to confluence, trypsinized, and seeded in the presence of 0.4 mg/ml tetracycline (Life Technologies, Inc.) to a very low density (1200) so that single clones could be isolated by picking the clones with sterile trypsin-embedded cotton-sticks. G418-resistant clones were continuously grown in the presence of G418 (0.4 mg/ml). Screening of positive clones expressing the sense or antisense human transcript was performed by Northern blot analysis using BamHI fragment of human rBAT cDNA cloned in pSPORT1 (1).

Northern blot analysis—Total RNA from OK cells was transferred to nylon membranes (Hybond N, Amersham) by capillarity in 10 × 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 after size separation on a 1.2% agarose/formaldehyde gel. RNA was transferred to nylon membranes (Hybond N, Amersham) by capillarity in 10 × 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

RESULTS

OK Cells Express the rBAT Gene—OK cells mRNA showed a transcript of 2.3 kb that hybridized with a human rBAT cDNA probe (data not shown). This agrees with previous studies that reported a transcript of similar size detected by Northern blot with rabbit or rat rBAT cDNA probes (27, 28). Cross-reaction with the homologous cell surface antigen 4F2hc transcript seems to be ruled out, since no hybridization signal was obtained in OK cells mRNA using a human 4F2hc cDNA (37 probe) (data not shown). Poly(A)⁺ RNA from OK cells expressed b²⁺⁺-like amino acid transport activity upon injection in Xenopus oocytes. Thus, injection of mRNA from OK cells in oocytes resulted in sodium-independent L-leucine uptake, which was inhibited (>-60%) by L-arginine (Fig. 1). Similarly, oocytes injected with mRNA from OK cells showed expression of sodium-independent uptake of 50 μM L-leucine (induced activity over background of 2.6 ± 0.7 pmol/30 min per oocyte) and 50 μM L-arginine (induced activity over background of 33.0 ± 4.3 pmol/30 min per oocyte), which were inhibited (>60%) by a 100-fold excess of L-leucine (data not shown). Hybrid expression of OK mRNA with an antisense oligonucleotide complementary to the human (and rat) rBAT cDNA specifically blocked (i.e. the sense oligonucleotide had no effect) expression of sodium-independent L-leucine uptake to the level inhibited by a 100-fold excess of L-arginine (Fig. 1). Similarly, OK mRNA expression of sodium-independent L-arginine uptake inhibited by L-leucine was hybrid-depleted (>90%) specifically by the same antisense oligonucleotide in oocytes (data not shown). These results demonstrate the functional expression of rBAT mRNA in OK cells. To provide further evidence for such expression, we searched for a partial sequence of rBAT cDNA in OK cells. RT-PCR from OK cell RNA, using oligonucleotides (C4D, sense; CSR, antisense; described in Ref. 8) complementary to the human rBAT cDNA, yielded a specific fragment of ~700 bp (data not shown). The nucleotide sequence of this fragment shows significant homology to human (74%), rabbit (74%), and rat (70%) rBAT cDNA (data not shown). The predicted amino acid sequence of this OK cell fragment covers amino acid residues 373–593 of the predicted human rBAT

![Figure 1](http://www.jbc.org/)

**Fig. 1.** rBAT antisense hybrid depletion of the L-arginine-inhibitable L-leucine uptake induced by OK cells mRNA in oocytes. Oocytes were injected with water containing 0 or 35 ng of OK cell mRNA. Prior to injection, mRNA was incubated alone (Control) or in the presence (20 μM) of a human rBAT antisense or sense oligonucleotide. Five days after injection, uptake of 50 μM L-leucine was determined for 30 min incubations and either in the absence or in the presence of 5 mM L-arginine. Each data point is the mean ± S.E. of the values (pmol/30 min/oocyte) of OK cell mRNA-induced L-leucine uptake, calculated by subtracting the uptake values obtained in water-injected oocytes from the uptake values in poly(A)⁺ RNA-injected oocytes. Uptake of L-leucine in water-injected oocytes were 7.3 ± 0.5 and 7.4 ± 0.3 pmol/30 min/oocyte in the absence or in the presence of L-arginine, respectively. Results were obtained with 6–8 oocytes per condition in a representative experiment. Another independent experiment showed similar results. The antisense effect and the L-arginine inhibition in the control and sense groups, but not in the antisense groups, were statistically significant (Student’s t-test; p < 0.01).
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protein and the second and third putative transmembrane
domains in the topology model proposed by Tate and co-work-
ers (25) (Fig. 2). Homology of this predicted protein fragment to
human, rabbit, and rat rBAT protein DNA ranged between 70% and
72% identity (84–86% similarity). Two out of three potential
N-glycosylation sites present in the human, rabbit, and rat
rBAT protein are conserved in the predicted OK cell rBAT
protein fragment (Fig. 2). Regions with maximal homology
between the four predicted protein fragments are those corre-
spending to the second and third putative transmembrane
domains and around the second N-glycosylation site of the
protein fragments. The region comprising the second putative
transmembrane domain corresponds to one of the fragments
highly conserved between rBAT and 4F2hc predicted proteins
(38). These results demonstrate that OK cells express the rBAT
gene.

OK Cells Express b0,+–like Amino Acid Transport Activity
in the Apical Pole—OK cells, grown on plastic, showed uptake of
L-cystine, L-arginine, and L-leucine in the absence of sodium
(Fig. 3). Most of the 50 μM L-cystine uptake was sodium-inde-
dependent (~80%) (MGA medium: 1.39 ± 0.07 nmol/mg
protein·min, n = 8; sodium medium: 1.87 ± 0.05 nmol/mg
protein·min, n = 8). This is in full agreement with a previous
report of L-cystine uptake in OK cells (31). Approximately half
(~55%) of the L-leucine uptake was sodium-independent (MGA
medium: 6.37 ± 0.09 nmol/mg protein·min, n = 3; sodium
medium: 11.5 ± 0.6 nmol/mg protein·min, n = 3). Uptake of
L-arginine was completely sodium-independent (MGA medium:
11.2 ± 0.5 nmol/mg protein·min, n = 5; sodium medium: 9.6 ±
1.1 nmol/mg protein·min, n = 5).

The sodium-independent uptake of L-cystine, and part of the
L-arginine and L-leucine uptake, showed a pattern of substrate
inhibition very similar to the b0,+–like transport activity elic-
ted by rBAT in oocytes (27). Thus, L-cystine uptake was almost
completely abolished by a 100-fold excess of L-arginine and
L-leucine in the uptake medium; in contrast, L-glutamate did
not affect L-cystine transport, precluding expression of system
Xc (Fig. 3). Similarly, the uptake of L-arginine was strongly
inhibited (70–80%) by a 100-fold excess of L-leucine (Fig. 3),
and the uptake of L-leucine was partially inhibited (60–70%)
by a 100-fold excess of L-arginine (Fig. 3). The non-metabolizable
amino acid analog BCH, a substrate model for system L, par-
cially inhibited L-leucine uptake (20–40%). This suggests that
part (≤30%) of L-arginine and L-leucine uptake is due to
transport activities resembling systems y+ and L, respectively.
On the other hand, interaction of L-leucine with sodium-inde-
dependent L-arginine uptake suggests system b0,+–like or system
y+ L transport activities (27, 38, 39). Inhibition of 50 μM L-
arginine uptake by varying concentrations of L-leucine (100 μM
to 10 mM) was identical in the absence and in the presence of
sodium (data not shown). This strongly indicates that the up-

FIG. 2. Comparison of the predicted and amino acid sequence of OK cell rBAT protein with the human, rabbit, and rat counterparts. The first line shows the OK cells rBAT amino acid sequence. Below, only substituted amino acids in the sequences of human (second line), rabbit (third line), and rat (fourth line) rBAT proteins are shown. Amino acid gaps in the sequence are indicated by dashes. This fragment of the OK cell rBAT protein is 220 amino acid residues long and in this alignment starts at the amino acid residue 373 of the human rBAT protein. This fragment of the OK cell rBAT protein is 72%, 71%, and 70% identical (85%, 86%, and 84% similarity) to the human, rabbit, and rat rBAT protein fragment, respectively. Two putative transmembrane domains, corresponding to the second and third domains of the four-transmembrane-domain model for the rBAT protein proposed by Tate and co-workers (27) are underlined in the OK cell protein sequence. Two (OK cell protein) and three (human, rabbit, and rat proteins) potential N-glycosylation sites are boxed.

FIG. 3. Sodium-independent uptake of amino acids in OK cells. Uptake of 50 μM L-[35S]cystine, L-[3H]arginine, or L-[3H]leucine by OK cells were measured in the absence (Control) or in the presence of the indicated unlabeled L-amino acids at 5 mM concentration in the uptake MGA medium. Uptake values (nmol/mg protein·min) are the mean ± S.E. from 5–12 determinations from two to four independent experiments. All the inhibitions shown were statistically significant (Student’s t-test at least p ≤ 0.01) with the exception of L-cystine transport in the presence of L-glutamate.
the uptake of L-arginine is not due to y'-L activity. Studies using L-cystine as inhibitor are difficult to perform due to the low solubility of this amino acid. For this reason we analyzed the effect of L-cystine on the uptake of L-arginine and L-leucine at low concentration. The uptake of 5 μM L-arginine (1.28 ± 0.06 nmol/mg protein·min) was inhibited (23%) by 450 μM L-cystine (0.99 ± 0.02 nmol/mg protein·min) and to a similar extent by 450 μM L-leucine (1.00 ± 0.09 nmol/mg protein·min). Similarly, the uptake of 5 μM L-leucine (0.73 ± 0.07 nmol/mg protein·min) was inhibited (27%) by 450 μM L-cystine (0.53 ± 0.01 nmol/mg protein·min). In all, these results show that, in OK cells, uptake of L-cystine is mostly due to the system b0,+ like activity, and that the uptake of L-arginine and L-leucine in the absence of sodium is due to both system b0,+ like and, probably, systems y' and L, respectively.

Kinetic analysis of L-cystine uptake, examined over a range of concentration from 5 μM to 450 μM, showed apparent Km values of 370 ± 40 μM and 13.6 ± 1.0 nmol/mg protein·min, respectively (data not shown). In agreement with a previous report on OK cells (31), one high affinity system appears to be present in OK cells. Kinetic analysis of L-arginine and L-leucine was performed over the range of 5 μM to 1000 μM. For arginine, one single kinetic component was observed with apparent kinetic parameters of Km = 211 ± 48 μM and Vmax = 59.9 ± 6.6 nmol/mg protein·min for L-arginine (data not shown). For L-leucine, the apparent kinetic parameters were: Km = 175 ± 56 μM and Vmax = 20.8 ± 2.5 nmol/mg protein·min (data not shown). These low apparent Km values for L-cystine, L-arginine, and L-leucine are of the same order (slightly higher) as those reported for these amino acids via the b0,+ like amino acid transport system elicited by human, rabbit, and rat rBAT cRNA in Xenopus oocytes (1, 2, 27, 28, 40).

Recently, it has been reported that system b0,+ like expressed by rBAT in oocytes shows trans-stimulation, suggesting that this transport system is an amino acid exchanger (3-5). To provide further evidence for the presence of system b0,+ like transport activity in OK cells, we searched for trans-stimulation of L-arginine efflux by different amino acids. Efflux was very low in the absence of amino acids (~1% of the total 2-min loading of L-[3H]arginine) but was increased by amino acid substrates of system b0,+ like in the external medium: 17-fold by 1 mM L-arginine, 6-fold by 1 mM L-leucine, and >2-fold by 200 μM L-cystine (Fig. 4). In contrast, L-arginine efflux was not trans-stimulated by 1 mM L-glutamate in the external medium (Fig. 4). The finding that most (~70%) of the L-arginine uptake in OK cells was due to system b0,+ like activity and that y'-L activity was not present in these cells indicates that trans-stimulation of L-arginine efflux by L-leucine is due to system b0,+ like. Similarly, L-leucine efflux was trans-stimulated by 1 mM L-arginine (6-fold) and by 300 μM L-cystine (2.5-fold) (data not shown), also showing trans-stimulation with characteristics of system b0,+ like.

Finally, we investigated the polarity of system b0,+ like activity in OK cells grown on transwell filters. Sodium-independent L-arginine (Fig. 5) and L-arginine (data not shown) uptake were higher (6-8-fold) in the apical pole. This is in full agreement with a previous report of L-cystine uptake on OK cells (31). Interestingly, the uptake of L-cystine and L-arginine in the apical but not in the basolateral pole showed the characteristic inhibition pattern of system b0,+ like; L-cystine uptake was abolished by a 100-fold excess of L-arginine (Fig. 5), and the uptake of L-arginine was strongly inhibited (~80%) by the 100-fold excess of L-leucine (data not shown). In all, these results demonstrate that OK cells express system b0,+ like amino acid transport activity in the apical pole.

Transfection of rBAT Antisense Results in a Specific Decrease of System b0,+ like Transport Activity in OK Cells—In order to demonstrate that the rBAT gene was responsible for system b0,+ like amino acid transport activity in OK cells, we performed rBAT cDNA antisense expression in these cells. After transfection with 5'-end fragments of human rBAT cDNA antisense or sense and Geneticin selection, cell clones were searched for expression of the transfected constructs. Two clones were positive for antisense transcript expression, and two others for sense expression (data not shown). Expression of endogenous rBAT mRNA in these clones was studied with the PCR-amplified fragment rBAT cDNA from OK cells shown in Fig. 2 as a probe. In comparison with control (i.e. untransfected cells), the rBAT mRNA levels were lower in two antisense
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Figure 6. A, Northern blot analysis for human rBAT-sense and antisense expression and for the endogenous rBAT mRNA in transfected OK cells. A human rBAT cDNA probe hybridized to a transcript of ~1.2 kb in length, which is present in the RNA from OK cells transfected with rBAT-sense or rBAT-antisense, but absent in nontransfected cells (OK) (left panel). 30 µg of total OK cell RNA was loaded in each lane. An OK cell rBAT cDNA probe, corresponding to the PCR-amplified cDNA fragment shown in Fig. 2, hybridized with a transcript of ~2.3 kb present in nontransfected OK cells (control) and in rBAT-sense and rBAT-antisense OK transfected cells total RNA (middle panel). 30 µg of OK cell total RNA was loaded per lane. The level of rBAT mRNA was reduced in the OK cells transfected with rBAT-antisense to ~20% of that in control and rBAT-sense groups, as revealed by scanning densitometry in two independent Northern blot analyses. Ethidium bromide staining of the Northern blot membrane shown in the middle panel (right panel). B, amino acid uptake in OK cells transfected with human rBAT sense or antisense. Uptake of 50 µM L-[3H]cystine, L-[3H]arginine, or L-[3H]leucine by OK cells was measured in the absence or in the presence of the indicated unlabeled l-amino acids or the amino acid analog BCH at 5 mM concentration in the uptake MGA medium. Uptake values (nmol/mg protein/min) correspond to the uptake inhibited by the indicated amino acids or analog and are the mean ± S.E. from triplicates of a representative experiment. In contrast to system b0,−-like transport activity, the uptake of L-leucine inhibited by the amino acid analog BCH, model for system L, was unaffected in the AS1 antisense clone (Fig. 6B). Similarly, the sodium-dependent uptake of 50 µM L-leucine was not affected in the antisense AS1 clone (i.e. the uptake values from triplicates of a representative experiment were: 4.7 ± 1.4 and 4.0 ± 0.7 nmol/mg protein/min for the S1 sense and the AS1 antisense clones, respectively). This demonstrates that rBAT antisense expression in OK cells results in a specific decrease in system b0,−-like transport activity. Kinetic analysis of L-cystine uptake showed that rBAT antisense expression reduces the apparent Vmax (7.8 ± 0.8 and 3.5 ± 0.7 nmol/mg protein/min, for the S1 sense and the AS1 antisense clones, respectively) without a significant effect on the estimated apparent Km values (227 ± 39 and 285 ± 50 µM, for the S1 sense and the AS1 antisense clones, respectively) (Fig. 7).

Discussion
Here we have shown that system b0,−-like is the major component of the transport of cystine in the apical pole of the opossum kidney cell line OK. This system is very similar to the exchanger activity of amino acids elicited by rBAT cRNA in oocytes. In addition, we have demonstrated that rBAT expression is necessary for this b0,−-like amino acid transport activity in this renal epithelial cell line. Due to the role of human rBAT gene in type I cystinuria (8, 10, 12), a corollary of the present study is that the b0,−-like amino acid exchanger would be defective in this type of cystinuria.

Identification of the amino acid transport activity associated with rBAT in renal cells has so far been elusive. No functional

clones (AS1 and AS2: ~20% and ~50% of control values, respectively) and unaffected in the two sense clones (S1 and S2). Expression of the transfected constructs and the rBAT mRNA levels in antisense AS1 and sense S1 clones, as well as in control cells (i.e. untransfected) are shown in Fig. 6A. An initial screening for amino acid transport activity in these clones revealed a decreased L-arginine transport activity in the antisense clones, which showed reduced rBAT mRNA expression. Thus, 50 µM L-arginine uptake in MGA uptake medium, expressed as nmol/mg protein-min, was: 11.0 ± 0.5 for control untransfected cells, 10.5 ± 0.3 and 9.5 ± 0.5 for S1 and S2 sense clones, and 6.0 ± 0.4 and 6.6 ± 0.3 for AS1 and AS2. Similar results were obtained for L-leucine uptake (data not shown).

Cell clones AS1 (antisense) and S1 (sense) were used for further experiments in which system b0,−-like activity was studied. Consistently (i.e. from passage 20 to 22), b0,−-like transport activity was lower (40–55%) in the AS1 antisense clone than in the S1 sense clone (Fig. 6B). System b0,−-like transport activity was almost identical in the S1 sense clone and in control untransfected cells (Figs. 3 and 6B). The b0,−-like transport activity expressed by rBAT cRNA in oocytes shows hetero-exchange between L-arginine and L-leucine (3–5). This has also been suggested for this transport activity in OK cells in the present study (Fig. 4). In order to demonstrate that rBAT is responsible for this activity via b0,−-like transporter, trans-stimulation of L-arginine efflux by L-leucine was studied in AS1 antisense and S1 sense clones. This trans-stimulation was reduced to ~50% in the AS1 antisense clone. Thus, efflux (cpm/mg protein-min, corrected for the radioactivity loading after 2 min of 50 µM L-[3H]arginine of 85,000 cpm/mg protein) was 1,300 ± 1,100 and 1,200 ± 600 in the presence of 1 mM L-glutamate in the medium for S1 sense and AS1 antisense clones, respectively. This efflux was trans-stimulated to 17,500 ± 900 and 9,200 ± 300 by 1 mM L-leucine in the medium of S1 sense and AS1 antisense cells, respectively (data are mean ± S.E. of triplicates from a representative experiment). In contrast to system b0,−-like transport activity, the uptake of L-leucine inhibited by the amino acid analog BCH, model for system L, was unaffected in the AS1 antisense clone (Fig. 6B).

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Identification of the amino acid transport activity associated with rBAT in renal cells has so far been elusive. No functional
expression of the amino acid transport activity associated with rBAT has been obtained in mammalian cells (e.g. COS cells) (30). All the data previously reported on the amino acid transport activity associated with rBAT were obtained in Xenopus oocytes (reviewed in Ref. 41). In addition, the rBAT protein has a low predicted number of transmembrane domains (25, 27, 28), and indirect evidence suggested its association with an unidentified subunit of 35–50 kDa to give a putative functional complex of 125 kDa in kidney and in oocytes expressing rBAT (Ref. 29). All this prompted the hypothesis that rBAT is not an amino acid transporter by itself, but rather full expression of the b0,1-like exchanger can only be achieved by association with an endogenous subunit of the oocyte. In this situation, description of the amino acid transport activity associated with rBAT in renal cells was a clear prerequisite for the full understanding of the physiology of rBAT. The present study demonstrates that, as in oocytes, rBAT expression is necessary for the b0,1-like transport activity present in the OK cell line. Whether rBAT acts as a modulator or as a catalytic component of this transport activity remains to be established through reconstitution experiments and identification of the putative subunit linked to rBAT.

To our knowledge the present study represents the first description in renal epithelial cells of the b0,1-like amino acid transport activity, defined as high affinity sodium-independent heteroexchange diffusion for cystine, dibasic (e.g. arginine) and neutral (e.g. leucine) amino acids. Only fragmental views of this amino acid transport activity have been described in kidney and intestine. Thus, functional studies indicated a high affinity reabsorption system for L-cystine in the proximal straight tubule of the nephron (23, 24) (i.e. S3 segment, where rBAT has been localized; Refs. 6 and 7). This was also shown to be present in the small intestinal mucosa and to be defective in biopsies from patients with cystinuria (20, 21, 42). This high affinity system is shared with dibasic amino acids and shows heteroexchange diffusion of dibasic amino acids and cystine (17–19, 42). Transport of L-cystine, examined at low (μM) concentration, is inhibited by some L-neutral amino acids, suggest-
excretion higher than the cystine that reaches the kidney for glomerular filtration.

Finally, the OK cells could be envisaged as an ideal model with which to study the cell biology and regulation of this transport activity associated with rBAT. The b0,1-glomerular filtration.

ing the rBAT antisense and sense constructs and Robin Rycroft for about the hormonal regulation of this reabsorption system for acid exchanger seems to be the only transport system for cys-

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