Relació estructura-funció en la família de transportadors d'aminoàcids heteromultimèrics. Identificació d'una nova família de transportadors lisosomals

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RELACIÓ ESTRUCTURA-FUNCIÓ EN LA FAMÍLIA DE TRANSPORTADORS D'AMINOÀCIDS HETEROMULTIMÈRICS IDENTIFICACIÓ D'UNA NOVA FAMÍLIA DE TRANSPORTADORS LISOSOMALS

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Barcelona, desembre de 1999

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 28, Issue of July 9, pp. 19738–19744, 1999
Printed in U.S.A.
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Identification of a Membrane Protein, LAT-2, That Co-expresses with 4F2 Heavy Chain, an L-type Amino Acid Transport Activity with Broad Specificity for Small and Large Zwitterionic Amino Acids*

(Received for publication, , March 22, 1999, and in revised form, April 16, 1999)

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We have identified a new human cDNA, L-amino acid transporter-2 (LAT-2), that induces a system L transport activity with 4F2hc (the heavy chain of the surface antigen 4F2, also named CD98) in oocytes. Human LAT-2 is the fourth member of the family of amino acid transporters that are subunits of 4F2hc. The amino acid transport activity induced by the co-expression of 4F2hc and LAT-2 was sodium-independent and showed broad specificity for small and large zwitterionic amino acids, as well as bulky analogs (*e.g.* BCH (2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid)). This transport activity was highly *trans*-stimulated, suggesting an exchanger mechanism of transport. Expression of tagged N-*myc*-LAT-2 alone in oocytes did not induce amino acid transport, and the protein had an intracellular location. Co-expression of N-*myc*-LAT-2 and 4F2hc gave amino acid transport induction and expression of N-*myc*-LAT-2 at the plasma membrane of the oocytes. These data suggest that LAT-2 is an additional member of the family of 4F2 light chain subunits, which associates with 4F2hc to express a system L transport activity with broad specificity for zwitterionic amino acids. Human LAT-2 mRNA is expressed in kidney >>> placenta >> brain, liver > spleen, skeletal muscle, heart, small intestine, and lung. Human *LAT-2* gene localizes at chromosome 14q11.2–13 (13 cR or ~286 kb from marker D14S1349). The high expression of LAT-2 mRNA in epithelial cells of proximal tubules, the basolateral location of 4F2hc in these cells, and the amino acid transport activity of LAT-2 suggest that this transporter contributes to the renal reabsorption of neutral amino acids in the basolateral domain of epithelial proximal tubule cells.

Last year, three amino acid transporter cDNAs (LAT-1, y^+LAT-1, and y^+LAT-2)[1] were identified as subunits of the heavy chain of the cell surface antigen 4F2 (4F2hc, also named CD98) (1–3). These subunits co-express amino acid transport activity with 4F2hc in oocytes (*i.e.* system L for LAT-1, and system y^+L for y^+LAT-1 and y^+LAT-2) (1–4). The role of this family of proteins in amino acid transport has recently been demonstrated by the fact that mutations in the *y^+LAT-1* gene cause lysinuric protein intolerance, an inherited amino aciduria due to a defective renal reabsorption mechanism of dibasic amino acids (5, 6). The structural and functional similarities between 4F2hc and its homologous protein rBAT suggest that a member of this family of subunits might be the subunit of rBAT needed to fully express the amino acid transport system $b^{0,+}$ activity (reviewed in Refs. 7 and 8). After the identification of *rBAT* as the Type I cystinuria gene (9), this subunit is a good candidate for non-Type I cystinuria (7). A search throughout gene data bases suggests that there may be as many as four new human members of the family of subunits of 4F2hc and rBAT.

Kanai and co-workers (1) identified rat LAT-1 (also known as TA1) by co-expression cloning with 4F2hc in oocytes. The co-expressed transport activity shows clear characteristics of the amino acid transport system L: high affinity (K_m in the low μM range), sodium-independent, and *trans*-stimulated transport for large zwitterionic amino acids. Some of these characteristics have also been demonstrated for the human (E16, Ref. 2) and *Xenopus laevis* orthologs of LAT-1 (ASUR4, Ref. 2; IU12, Ref. 3). System L is almost ubiquitous (10), and variants of system L have been described (11, 12). The expression of rat LAT-1 is not ubiquitous, and it is not present in tissues such as kidney and liver (1), which suggests that homologs of LAT-1 might encode system L amino acid transporter variants.

In this study we have identified the fourth human member (LAT-2) of this family of amino acid transporters. LAT-2 does not induce transport of amino acids in oocytes when it is injected alone, but a variant of system L transport activity (*i.e.* with broad specificity for small and large zwitterionic amino acids) is co-expressed when LAT-2 is injected with 4F2hc. We demonstrate here that co-expression of LAT-2 with 4F2hc brings the former to the oocyte plasma membrane. Its expres-

* This work was supported in part by Dirección General de Investigación Científica y Técnica Research Grant PM96/0060 and by Grant 1995 SGR 537 from the Generalitat de Catalunya (Spain).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™/EBI Data Bank with accession number(s) AF135828, AF135829, AF135830, and AF135831.

‡ These two authors contributed equally to this study.

§ Recipients of a grant from the Comissió Interdepartamental de Recerca i Innovació Tecnològica, Catalonia, Spain.

¶ Recipients of a grant from the Ministerio de Educación y Cultura, Spain.

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[1] The abbreviations used are: LAT, L-amino acid transporter; y^+LAT, y^+ L-amino acid transporter; 4F2hc, heavy chain of the cell surface antigen 4F2; BCH, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid; rBAT, related to $b^{0,+}$ amino acid transporter; PCR, polymerase chain reaction; ORF, open reading frame; nt, nucleotide; bp, base pair(s); kb, kilobase or kilobase pair(s); TBS, Tris-buffered saline; SHGC, Stanford Human Genome Center; EST, expressed sequence tag.

This paper is available on line at http://www.jbc.org

sion in the epithelial cells of the proximal tubule suggests a role of LAT-2/4F2hc in the renal reabsorption of neutral amino acids.

EXPERIMENTAL PROCEDURES

PCR Amplification, Sequencing, and LAT-2 cDNA Construction—For PCR amplification, first-strand cDNA was synthesized from 5 μg of total RNA purified from opossum kidney (13) cells using SuperScript II kit (Life Technologies, Inc.). Two degenerate forward and reverse primers were designed based on two highly conserved regions among the first known members of this family of amino acid transporters. PCR amplification, subcloning into pGEM-T easy vector (Promega), and sequencing were carried out as described elsewhere (3).

The open reading frame (ORF) of LAT-2 was obtained from two partial human LAT-2 cDNA clones (IMAGE No. 322502 and No. 267204). Clone 322502 was cut with *Not*I and *Avr*II to create the 5′-end fragment of LAT-2 (from nt 1 to 1152 of the LAT-2 cDNA). Clone 267204 was digested with *Eco*RI and *Avr*II to create the 3′-end fragment of the LAT-2 coding sequence (nt 1152 to 3′-end of the LAT-2 cDNA) ligated to the pT7T3D vector. Both fragments were ligated to create a LAT-2 cDNA fragment covering the ORF (5′-end to nt 2050; see Fig. 1) in pT7T3D vector. To improve expression in oocytes, an *Ssp*I-*Not*I fragment of LAT-2 was cloned into pNKS2-*myc* *Not*I vector (a gift from G. Schmalzing; Ref. 14). To create an N-*myc*-tagged LAT-2 cDNA, pT7T3D-LAT-2 was PCR-amplified with primers M13 forward (16-mer) and 5′-ACGTCTAGTCGACATGGAAGAAGGAGCCAGGCAC-3′ (containing a *Sal*I site and the first 21 nt of the ORF of LAT-2). The PCR product was digested with *Sal*I and *Not*I. The resulting fragment of LAT-2 was cloned into pNKS2-*myc* *Not*I. The N-*myc*-tagged LAT-2 cDNA was tested by sequencing. All sequences carried out in this work were performed in both directions with d-rhodamine dye terminator cycle sequencing ready reaction kit (Perkin-Elmer). The sequence reactions were analyzed with an Abi Prism 377 DNA sequencer.

Oocytes, Injections, and Uptake Measurements—Oocyte origin, management, and injections were as described elsewhere (15, 16). Defolliculated stage VI *X. laevis* oocytes were injected with 10 ng/oocyte human 4F2hc, human LAT-2, N-*myc*-LAT-2, or *X. laevis* IU12 cRNA. Synthesis of human 4F2hc cRNA (17) was as described (18). *X. laevis* LAT-1 (*i.e.* IU12) was a gift from Y. B. Shi (19), and the cRNA was synthesized as described elsewhere (3). Human LAT-2 cRNA was obtained by cutting the cDNA with *Not*I and using T7 polymerase.

Influx rates of L-[^{3}H]arginine, L-[^{3}H]leucine, L-[^{3}H]alanine, and L-[^{3}H]glutamine (Amersham Pharmacia Biotech) were measured in 100 mM NaCl or 100 mM CholineCl medium at the indicated number of days after injection and under linear conditions as described (15). Amino acid transport rates obtained with oocytes injected with water (50 nl) were similar to those of noninjected oocytes (data not shown). For L-[^{3}H]isoleucine efflux measurements, groups of five cRNA-injected or noninjected oocytes were incubated with 50 μM L-[^{3}H]isoleucine (3 μCi/μl) for the indicated period of time (see legend to Fig. 7). Efflux was measured as described elsewhere (20).

Computer Analysis—Amino acid or nucleotide sequence homology search and the prediction of transmembrane segments of LAT-2 were performed as indicated elsewhere (3).

Northern Blot Analysis—A human adult poly(A$^+$) membrane from CLONTECH (Palo Alto, CA) was used. The insert of clone 267204 was separated from the pT7T3D vector by *Not*I-*Eco*RI digestion. This ~1-kb DNA fragment was purified, labeled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech) using a random oligonucleotide-priming labeling kit (Amersham Pharmacia Biotech), and used as a probe. Hybridization and washing conditions were as recommended by CLONTECH. In these conditions, y+LAT-1 and y+LAT-2 cRNAs were not detected (data not shown).

In Situ Hybridization—Sense and antisense cRNA probes were labeled with digoxigenin-11-UTP (Roche Molecular Biochemicals) by transcription of a LAT2 fragment (1–310 nt of the contig shown in Fig. 1) contained in the pT7T3D vector. The transcription reactions were set up at room temperature by mixing 7.5 μl of double-distilled water treated with diethyl pyrocarbonate, 1 μl of linearized template cDNA (1 μg), 4 μl of 5× transcription buffer (Promega), 2 μl of NTPmix (10 mM ATP, CTP, GTP, 6.5 mM UTP, 3.5 mM digoxigenin-11-UTP, Roche Molecular Biochemicals), 1 μl of RNAsin (30.6 units/μl, Amersham Pharmacia Biotech), and 2 μl of RNA polymerase (T7 or T3, 15 units/μl, Promega). Labeling reactions were performed at 37 °C for 2 h and stopped by incubation with 2 μl of RNase-free DNase (10 units/μl, Stratagene) for 15 min at 37 °C. cRNA fragments were precipitated overnight with 1/10 vol of 4 M LiCl and 2.5 volumes of ethanol at −80 °C. The precipitated cRNA was recovered in 10 μl of double-distilled water treated with diethyl pyrocarbonate.

Fresh human kidney was fixed in 4% paraformaldheyde, 0.1 M phosphate buffer and kept at 4 °C before use. Thereafter, the sections were washed in 0.1 M phosphate buffer (2 h, room temperature) and dehydrated with 70, 90, and 100% alcohol, alcohol/xylene (v/v), and xylene (2 h for each). Pieces were embedded in paraffin. 5-μm sections were cut on a Leica RM 2135 microtome and mounted on silenized slides (Perkin-Elmer). Sections were deparaffined with xylene and hydrated with 100, 90, and 70% ethanol and double-distilled water treated with diethyl pyrocarbonate, permeabilized with proteinase K (Roche Molecular Biochemicals) (1 μg/ml) in Tris-EDTA buffer, pH 8 (3 min, 37 °C), 0.2 N HCl (20 min, room temperature), and washed twice in 2× SSC solution (30 min, room temperature). The hybridization step was carried out with a solution containing 50% formamide, 10% dextran sulfate, 2× SSC solution, 1× Denhardt's solution, 400 ng/μl denatured salmon sperm DNA, and denatured (4 min, 70 °C) sense or antisense probes (5 ng/μl) for 16 h at 42 °C in a moist chamber. The sections were then washed in 4× SSC solution containing 45% formamide (2 min, room temperature), 0.1× SSC (1 h, 37 °C), digested with 20 μg/ml RNase A (Roche Molecular Biochemicals) (30 min, room temperature), and washed in 0.1× SSC solution (5 min, room temperature). Sections were rinsed twice in Tris-buffered saline (TBS), pH 7.5 (10 min, room temperature), blocked with 1% bovine serum albumin in TBS (30 min, room temperature), and incubated overnight with an alkaline phosphatase-conjugated anti-digoxigenin antibody (1:500) (Biocell, Cardiff, UK). They were then washed in TBS, pH 7.5 (10 min, room temperature), and TBS, pH 9.5, containing 50 mM $MgCl_2$ (10 min, room temperature) and developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Roche Molecular Biochemicals). Slides were examined on an Olympus microscope.

Chromosome Mapping—Chromosome mapping was done using the Stanford Human Genome Center G3 radiation hybrid panel (medium resolution). DNA samples of this panel, along with total genomic DNA and pT7T3-249835 (used as a positive control), were PCR screened for the presence of the genomic sequences flanked by the primers 12D (5′-GGCATCTCTCTTCCTAATG-3′) and 7R (5′-GCCAATGCTCTCCTCAGT-3′), which are located in the 3′-untranslated region of the cDNA. PCR amplifications were carried out in a Perkin-Elmer 9600 thermocycler as described elsewhere (3). Amplification conditions were as follows: 35 cycles of denaturing (94 °C, 30 s), annealing (58 °C, 40 s), and extension (74 °C, 30 s). PCR results were transformed into zeros (for no amplification) and ones (for positive amplification) and submitted to the radiation hybrid mapping e-mail server at the Stanford Human Genome Center (SHGC). The resulting chromosomal location, referred to a SHGC marker, was obtained automatically via e-mail from this server.

Localization of LAT-2 Expression by Confocal Microscopy—Groups of five oocytes were prepared for immunofluorescence 2 days after injection with 10 ng/oocyte human 4F2hc or N-*myc*-LAT-2 cRNA, alone or in combination. Oocytes were placed in 500 mm^3 cryomolds (Tissue-Tek, Miles Inc., Elkhart, IN), sliced, fixed, and permeabilized as described elsewhere (18). Slices were incubated with monoclonal antibody 9E10 anti-*myc* (ATCC, Manassas, VA), diluted 1/500 in 10% phosphate-buffered saline, at room temperature for 1 h. Slices were washed three times in phosphate-buffered saline, incubated with 7.5 μg/ml Texas red-conjugated goat anti-mouse (Molecular Probes, Leiden, The Netherlands) at room temperature for 1 h, washed three times in phosphate-buffered saline, and mounted in Immunofluore (ICN, Madrid, Spain).

RESULTS

Our goal was to identify any new member of the amino acid transporter-related family expressed in the kidney and potentially involved in reabsorption of amino acids. For this purpose, reverse transcription-PCR amplification of total RNA from opossum kidney cells was performed with degenerated primers as described for the identification of y$^+$LAT-1 (3). Electrophoretic analysis of the PCR reaction showed one band of 286 bp, which was subcloned into pGEMT-easy vector and amplified in *Escherichia coli*. The deduced amino acid sequence of one clone (b2c2) showed a significant degree of identity to the amino acid transporter-related proteins: 46, 45, 43, 41, and 43% with human y$^+$LAT-1 and y$^+$LAT-2 and *Xenopus*, rat, and human LAT-1, respectively. This homology is compatible with the assumption that b2c2 is part of a cDNA corresponding to a

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CAACACTTTTCTTGTTTGTAAACGCGAGTGACCAGAAAGTGTGAATGCGGAGTAGGAATA   60
TTTTTCGTGTTCTCTTTTATCTGCTTGCCTTTTTTAGAGAGTAGCAGTGGTTCCTATTTC  120
GGAAAAGGACGTTCTAATTCAAAGCTCTCTCCCAATATATTTACACGAATACGCATTTAG  180
AAAGGGAGGCAGCTTTTGAGGTTGCAATCCTACTGAGAAGGATGGAAGAAGGAGCCAGGC  240
                                        M  E  E  G  A  R  H     7
ACCGAAACAACACCGAAAAGAAACACCCAGGTGGGGGCGAGTCGGACGCCAGCCCCGAGG  300
  R  N  N  T  E  K  K  H  P  G  G  G  E  S  D  A  S  P  E  A   27
CTGGTTCCGGAGGGGGCGGAGTAGCCCTGAAGAAAGAGATCGGATTGGTCAGTGCCTGTG  360
  G  S  G  G  G  G  V  A  L  K  K  E  I  G  L  V  S  A  C  G   47
GTATCATCGTAGGGAACATCATCGGCTCTGGAATCTTTGTCTCGCCAAAGGGAGTGCTGG  420
  I  I  V  G  N  I  I  G  S  G  I  F  V  S  P  K  G  V  L  E   67
AGAATGCTGGTTCTGTGGGCCTTGCTCTCATCGTCTGGATTGTGACGGGCTTCATCACAG  480
  N  A  G  S  V  G  L  A  L  I  V  W  I  V  T  G  F  I  T  V   87
TTGTGGGAGCCCTCTGCTATGCTGAACTCGGGGTCACCATCCCCAAATCTGGAGGTGACT  540
  V  G  A  L  C  Y  A  E  L  G  V  T  I  P  K  S  G  G  D  Y  107
ACTCCTATGTCAAGGACATCTTCGGAGGACTGGCTGGGTTCCTGAGGCTGTGGATTGCTG  600
  S  Y  V  K  D  I  F  G  G  L  A  G  F  L  R  L  W  I  A  V  127
TGCTGGTGATCTACCCCACCAACCAGGCTGTCATCGCCCTCACCTTCTCCAACTACGTGC  660
  L  V  I  Y  P  T  N  Q  A  V  I  A  L  T  F  S  N  Y  V  L  147
TGCAGCCGCTCTTCCCCACCTGCTTCCCCCCAGAGTCTGGCCTTCGGCTCCTGGCTGCCA  720
  Q  P  L  F  P  T  C  F  P  P  E  S  G  L  R  L  L  A  A  I  167
TCTGCTTATTGCTCCTCACATGGGTCAACTGTTCCAGTGTGCGGTGGGCCACCCGGGTTC  780
  C  L  L  L  T  W  V  N  C  S  S  V  R  W  A  T  R  V  Q  187
AAGACATCTTCACAGCTGGGAAGCTCCTGGCCTTGGCCCTGATTATCATCATGGGGATTG  840
  D  I  F  T  A  G  K  L  L  A  L  A  L  I  I  I  M  G  I  V  207
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  W  N  F  L  N  Y  V  T  E  E  L  V  D  P  Y  K  N  L  P  R  267
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  A  I  F  I  S  I  P  L  V  T  F  V  Y  V  F  A  N  V  A  Y  287
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  V  T  A  M  S  P  Q  E  L  L  A  S  N  A  V  A  V  T  F  G  307
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TCATCAACTACGTGGGCTTCATCAACTACCTCTTCTATGGGGTCACGGTTGCTGGACAGA 1440
  I  N  Y  V  G  F  I  N  Y  L  F  Y  G  V  T  V  A  G  Q  I  407
TAGTCCTTCGCTGGAAGAAGCCTGATATCCCCCGCCCCATCAAGATCAACCTGCTGTTCC 1500
  V  L  R  W  K  K  P  D  I  P  R  P  I  K  I  N  L  L  F  P  427
CCATCATCTACTTGCTGTTCTGGGCCTTCCTGCTGGTCTTCAGCCTGTGGTCAGAGCCGG 1560
  I  I  Y  L  L  F  W  A  F  L  L  V  F  S  L  W  S  E  P  V  447
TGGTGTGTGGCATTGGCCTGGCCATCATGCTGACAGGAGTGCCTGTCTATTTCCTGGGTG 1620
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  Y  W  Q  H  K  P  K  C  F  S  D  F  I  E  L  L  T  L  V  S  487
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  Q  K  M  C  V  V  V  Y  P  E  V  E  R  G  S  G  T  E  E  A  507
CTAATGAGGACATGGAGGAGCAGCAGCAGCCCATGTACCAACCCACTCCCACGAAGGACA 1800
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CCTTCCTGGCCTGGATGGTGTCTCCCAGCTTCCCCGCCCAGCTTGCCCACCCCATAGTTG 3360
GTGAGATGCCAAGTTTGGTCTGAGTTGTGACCCCTTCAGAGTAGATGCCCGGCAGGCTGG 3420
GGTTGGCCCCTGGAGGGTCAGGGGACCATCTTCTTATTCCCTCTTTTCTCATTCCTCCAA 3480
CTTCCTCCCCTCCTTCAATTATTTTTTTGTAAAGTTGATGCCTTACTTTTTGGATAAATA 3540
TTTTTGAAGCTGGTATTTCTATTTCTTTTGGATTTTTTTTAATGTAAGGTTGTTTTGGGG 3600
GATGGAGTTAGAACCTTAATGATAATTTCTTTCGTTTGGTGTAGGTTTTAGAGATTTGTT 3660
TTGTGGAGAGGTTTTTTTCTTTTGATGTAATAAAATTTAAAATGGAAATGAAAAAAAAAA 3720
AAAAAAAAAAAA                                                   3733
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FIG. 1. **Nucleotide and deduced amino acid sequence of LAT-2 cDNA.** The size of the cDNA contig is 3733 bp, containing a 5′-untranslated region of 221 bp followed by an ORF of 535 amino acids and a 3′-untranslated region of 1904 bp that contains a 23-bp long poly(A) tail. The stop codon (TGA) is indicated by a *star*. The possible polyadenylation signal is *underlined*. The sequences of the overlapping segments were identical in all three clones except for nt 1422. At this position, clone 267204 has a G, as in the ESTs N32639 and N31874, and the corresponding amino acid residue is Val^{401}. In contrast, clone 249835 has an A, and the corresponding amino acid is Ile^{401}. This is probably a polymorphism. In agreement with the majority of available EST sequences, the contig LAT-2 shown in this figure and the cRNA injected in this study has G at position 1422 (*i.e.* Val^{401}).

new member of this family. By using the same computer approach (BLAST and EST Cluster Assembly Machine) as we recently used for the identification of y^+LAT-1 (3), a human EST (W39098, IMAGE clone 322502) that shows homology with the b2c2 fragment (92% identity in the amino acid sequence) was identified. Subsequently, EST W39098 was used to identify two other ESTs from the same cluster (N23973 and H84042, *from* IMAGE *clones 267204 and 249835, respectively*). Sequences of these overlapping EST clones revealed a 3′-polyadenylated cDNA contig (LAT-2) of 3733 bp (Fig. 1). The first ATG codon lies within a good consensus initiation sequence (5′-GAAGG) (21). The ORF continues to the first stop codon (TGA) at base 1827 and codes for a protein of 535 amino acid residues with a predicted molecular mass of 58,577 Da. The nucleotide sequences of EST clones 322502, 267204, and 249835 and LAT-2 cDNA have been deposited in the GenBank℠/EBI data base (accession numbers AF135828, AF135829, AF135830, and AF135831, respectively).

A multiple sequence alignment of the predicted amino acid sequence of human LAT-2, LAT-1, y^+LAT-1, and y^+LAT-2 is shown in Fig. 2. Human LAT-2 shows an amino acid sequence identity of 50, 44, and 45% to human LAT-1, y^+LAT-1 and y^+LAT-2, respectively. Hydrophobicity studies show 12 transmembrane domains with both C- and N-terminal segments intracytoplasmatic, which is the same protein structure suggested for the other members of this family (1–3, 19). Only the consensus for the position of the transmembrane segment III can vary for the proteins presented in Fig. 2. There is only one putative N-glycosylation site (Fig. 2, *boxed*) between the putative transmembrane segments VIII and IX. In our predicted model this segment is cytoplasmic and cannot be glycosylated. This finding is in full agreement with previous expression studies with rat and human LAT-1 (1–2). 4F2hc is associated with its subunits in a disulfide bond-dependent manner (2–4, 22) through cysteine residue 109 of human 4F2hc (18) and cysteine residue 164 of *Xenopus* LAT-1 (23). This cysteine residue is conserved in all human 4F2 light chains including LAT-2 (cysteine residue 154) (Fig. 2).

The human *LAT-2* gene was chromosome-mapped by using a radiation hybrid panel (see "Experimental Procedures") with primers corresponding to the 3′-untranslated region of the LAT-2 cDNA. From this screening we obtained 16 positive and 66 negative results. Chromosome mapping results, obtained from the SGHC server, linked Lat-2, with a logarithem odds score of 12.6, to a distance of 13 cR (286 kb) from the marker SHGC-13507 (D14S1349). The nearest centromeric marker to this one, marker SHGC-6999 (*X52889*), is located at chromosome 14q11.2–13.

*cRNA from LAT-2 was injected into oocytes alone or in combination with an equimolar quantity of human 4F2hc cRNA and tested for amino acid transport (Fig. 3). 4F2hc alone induced, as previously reported (16, 18, 24–27), y^+L amino acid transport activity (*i.e.* sodium-independent L-arginine transport and sodium-dependent L-leucine transport). LAT-2 alone induced weakly sodium-independent L-leucine transport. Interestingly, when 4F2hc and LAT-2 were co-injected, the induction of L-arginine transport was lower than that induced by 4F2hc alone, whereas the induction of sodium-independent L-leucine transport increased dramatically (Fig. 3). From four independent experiments the average co-expression of L-leucine transport relative to the induction of 4F2hc alone was 30-fold (ranging from 6- to 100-fold). The co-expression of leucine transport by 4F2hc and LAT-2 is sodium-independent, suggesting induction of a system L-type amino acid transport activity (Fig. 3). Kinetic analysis revealed an apparent K_m of 221 ± 54 μM for the transport of L-leucine induced by 4F2hc/

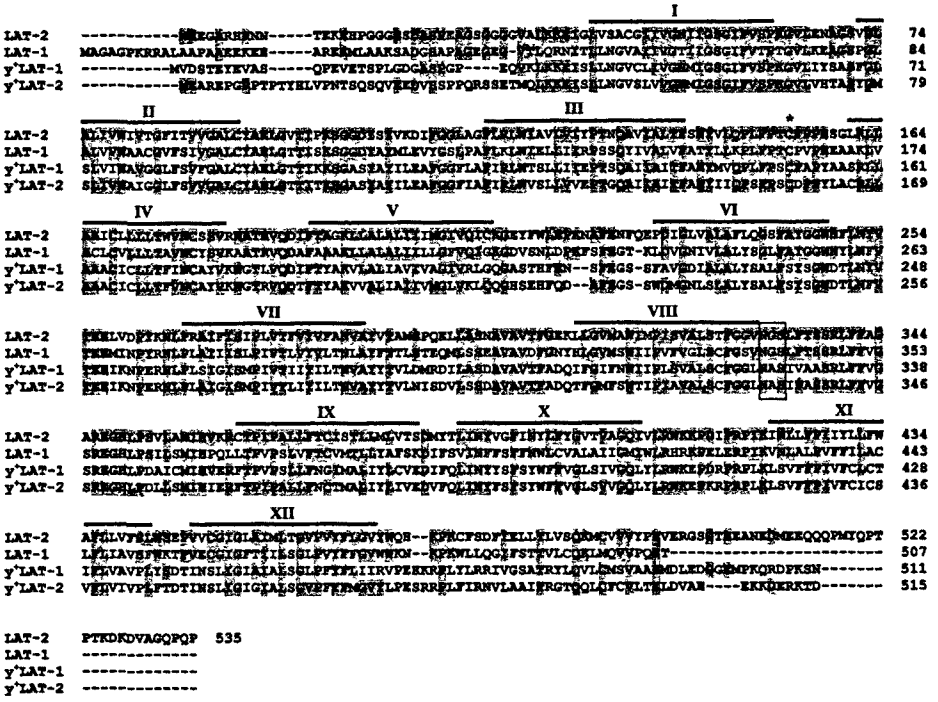

FIG. 2. **Amino acid sequence comparison of the four human members of the family of amino acid transporters that are subunits of 4F2hc.** Multialignment was done using the program CLUSTALW Sequence Alignment from the Baylor College of Medicine. The *thin horizontal lines* indicate the 12 putative transmembrane domains determined by computer analysis (see "Experimental Procedures"). The amino acid residues identical to LAT-2 sequence are indicated in *gray boxes*. The *solid frame box* indicates a potential N-glycosylation site, but according to our membrane topology prediction, this site is intracellular and cannot be glycosylated. The conserved cysteine residue is indicated with a *star*.

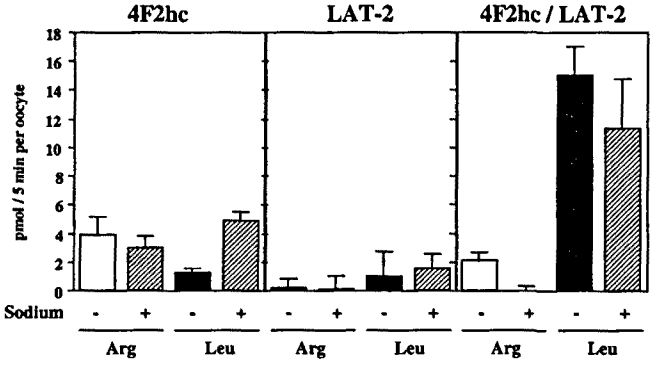

FIG. 3. **Co-expressed amino acid transport activity by 4F2hc and LAT-2.** Oocytes were injected with LAT-2 cRNA alone or in combination with human 4F2hc cRNA. Three days after the injection, the uptake of 50 μM L-[^{3}H]arginine (*Arg*) and 50 μM L-[^{3}H]leucine (*Leu*) in the presence (+, *slashed bars*) or absence (−, *open* or *closed bars*) of 100 mM NaCl was determined for 5 min. Transport of L-[^{3}H]leucine in the absence of sodium is *highlighted* in the *closed bars*. Amino acid uptake rates (pmol/5 min per oocyte) were calculated by subtracting the uptake of the noninjected group from that of the cRNA-injected groups. The amino acid uptake activity of uninjected oocytes was as follows: L-[^{3}H]arginine uptake, 3.0 ± 0.2 (choline medium) and 4.0 ± 0.7 (sodium medium); L-[^{3}H]leucine uptake, 1.8 ± 0.2 (choline medium) and 3.3 ± 0.4 (sodium medium). Data (mean ± S.E.) correspond to a representative experiment with 7–8 oocytes per group.

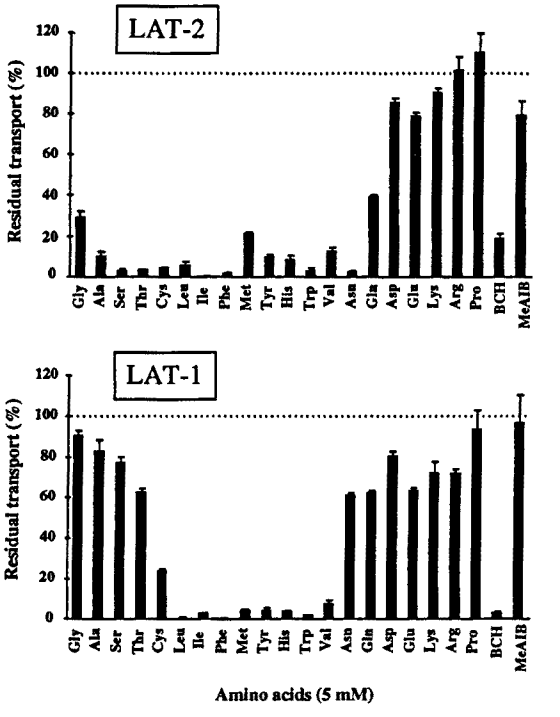

FIG. 4. **Inhibition pattern of the amino acid transport activity co-expressed by 4F2hc and LAT-2.** Two or 3 days after injection of 4F2hc cRNA together with LAT-2 or LAT-1 cRNA, the uptake of 50 μM L-[^{3}H]leucine in the absence of sodium was measured for 5 min in the absence (Control) or in the presence of the indicated amino acids or analogs at 5 mM. The expressed transport (*i.e.* subtracting transport of noninjected oocytes) in control groups was 15.3 ± 1.5 and 26.6 ± 4.0 pmol/5 min per oocyte for 4F2hc/LAT-2- and 4F2hc/LAT-1-injected oocytes, respectively. Data (mean ± S.E.) represent percentages of the amino acid residual transport in the presence of inhibitors. Transport of L-[^{3}H]leucine in noninjected oocytes was 1.6 ± 0.2 pmol/5 min per oocyte. Data correspond to two to four independent experiments, in which 7–8 oocytes were used per group in each experiment.

LAT-2 (data not shown).

To further characterize the uptake activity co-expressed by LAT-2 and 4F2hc, we measured the inhibition of sodium-independent leucine uptake by different amino acids at a 100-fold excess concentration (5 mM). Fig. 4 shows the inhibition pattern for the transport activity induced by LAT-2 and 4F2hc compared with that induced by *X. laevis* LAT-1 and 4F2hc. These results showed clearly that the transport activity induced by 4F2hc/LAT-2 and 4F2hc/LAT-1 is restricted to zwitterionic amino acids. The pattern of inhibition in the case of *X. laevis* LAT-1, restricted to large zwitterionic amino acids and analogs (*i.e.* BCH), is in full agreement with the pattern described for rat LAT-1 (1). In contrast, 4F2hc/LAT-2-induced transport activity was also practically abolished by small zwitterionic amino acids (*i.e.* glycine, alanine, serine, threonine, and cysteine), and it is clearly inhibited by glutamine and asparagine. To demonstrate transport of small zwitterionic amino acids via

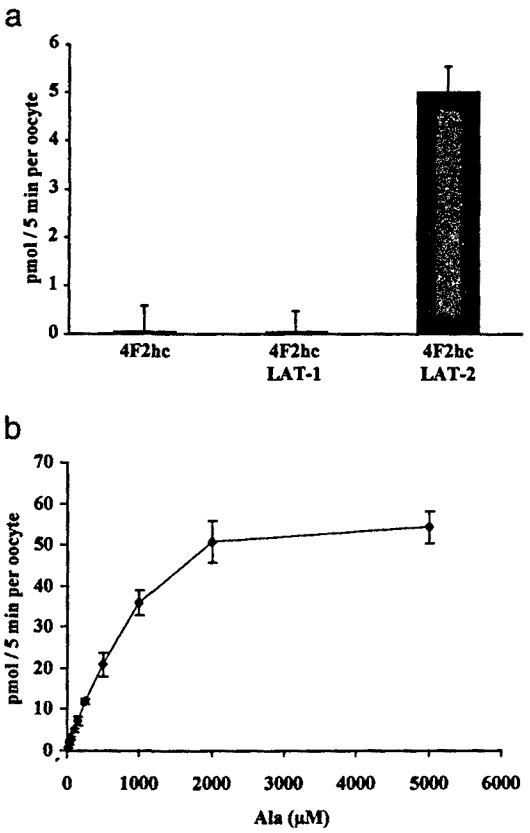

FIG. 5. **4F2hc/LAT-2 co-expresses alanine transport in oocytes.** *a*, oocytes were injected with 4F2hc cRNA alone or in combination with LAT-1 or LAT-2 cRNA. Three days after the injection, the uptake of 50 μM L-[^{3}H]alanine in the absence of sodium was determined for 5 min. Transport of noninjected oocytes was 6.5 ± 1.1 pmol/5 min per oocyte. Data (mean ± S.E.) correspond to a representative experiment with 7–8 oocytes per group. Another two independent experiments showed similar results. *b*, kinetic analysis of L-alanine transport co-expressed by 4F2hc/LAT-2. Oocytes were injected with 4F2hc and LAT-2 cRNA. Two days after the injection, the uptake of L-[^{3}H]alanine in the absence of sodium was determined for 5 min at different substrate concentrations (10, 25, 50, 100, 150, 250, 500, 1000, 2000, and 5000 μM). The transport activity level in noninjected oocytes was subtracted from that of cRNA-injected oocytes. Data (mean ± S.E.) correspond to a representative experiment with 5–6 oocytes per group. Kinetic parameters were: V_{max} = 64 ± 7 pmol/5 min per oocyte, K_m = 978 ± 143 μM, and *r* (correlation coefficient) = 0.92 ($p \leq 0.001$).

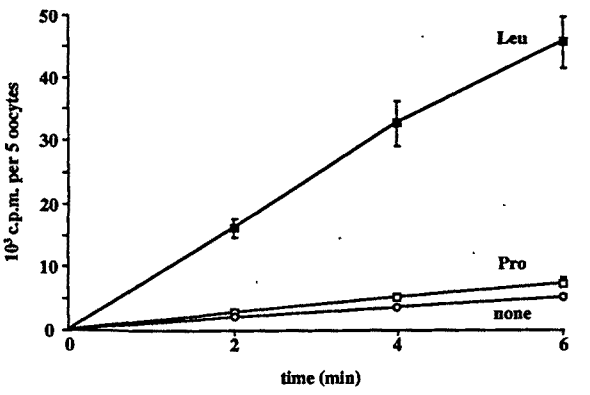

FIG. 6. ***Trans*-stimulation of efflux via 4F2hc/LAT-2 system L.** Oocytes were injected with 4F2hc cRNA alone or in combination with LAT-2 cRNA. Three days after the injection, oocytes were loaded with 50 μM L-[^{3}H]isoleucine for 60 min reaching the following uptake level of radioactivity: 21900 ± 1900, 3700 ± 400, and 2200 ± 400 cpm per oocyte in 4F2hc/LAT-2-injected, 4F2hc-injected, and noninjected oocytes, respectively. The efflux of radioactivity was then measured in the indicated periods of time in media containing 1 mM L-leucine (*Leu*, ■) or proline (*Pro*, □) or no amino acids (*none*, ○). Data (mean ± S.E.) correspond to a representative experiment with three groups of 5 oocytes per data point. The efflux rates in 4F2hc-injected and noninjected oocytes in the presence of 1 mM L-leucine in the medium were 1630 ± 200 and 1350 ± 100 × 10^3 cpm/5 oocytes per min, respectively. These efflux rates are indistinguishable from those of 4F2hc/LAT-2-injected oocytes in medium containing 1 mM proline or no amino acids.

this variant of system L, the uptake of 50 μM L-[^{3}H]alanine was determined in oocytes expressing 4F2hc/LAT-2 or 4F2hc/LAT-1. Interestingly, co-expression of 4F2hc/LAT-2 in oocytes, but not of 4F2hc/LAT-1, resulted in the induction of L-alanine transport above background (*i.e.* noninjected or 4F2hc-injected oocytes) (Fig. 5*a*). Kinetic analysis of this transport revealed an apparent K_m of 978 ± 142 μM (Fig. 5*b*). Similarly to alanine, 4F2hc/LAT-2 induced sodium-independent L-glutamine transport. Two days after injection, the induced uptake of 200 μM L-[^{3}H]glutamine was 0.3 ± 0.4 and 34.6 ± 4.0 pmol/5 min per oocyte for 4F2hc- and 4F2hc/LAT-2-injected oocytes, respectively. All of the above suggests that 4F2hc/LAT-2 represents a broad specificity variant of system L transporter for small and large zwitterionic amino acids.

In agreement with previous reports for the transport activity induced by 4F2hc in oocytes (18, 20) by LAT-1/4F2hc (1–2) and y^+LAT-1/4F2hc (4), LAT-2/4F2hc showed a high level of *trans*-stimulation. Fig. 6 shows that the efflux of L-[^{3}H]isoleucine in oocytes expressing LAT-2/4F2hc is dependent on the presence of a substrate in the medium (*e.g.* leucine), but it is not *trans*-stimulated by amino acids that are not substrates (*e.g.* proline) of LAT-2/4F2hc amino acid transporter. The level of efflux in $trans_0$ conditions (no amino acid substrates in the medium) in oocytes expressing LAT-2/4F2hc is identical to that of noninjected oocytes or oocytes expressing 4F2hc alone (Fig. 6). This result suggests a high level of exchanger coupling via LAT-2/4F2hc.

Recently, it has been shown that y^+LAT-1, LAT-1, and SPRM1 form a disulfide bond heterodimeric complex with 4F2hc (2–4, 22, 23). Moreover, Verrey and co-workers (2) have shown that 4F2hc brings SPRM1 to the oocyte plasma membrane. Fig. 7 shows that 4F2hc also brings LAT-2 to the oocyte plasma membrane. To follow the expression of LAT-2, a tagged LAT-2 cRNA (N-*myc*-LAT-2) was expressed in oocytes. N-*myc*-LAT-2 co-expresses with 4F2hc L-transport activity, but N-*myc*-LAT-2 alone does not induce amino acid transport activity (see legend to Fig. 7). Confocal immunofluorescence detected N-*myc*-LAT-2 at the oocyte plasma membrane when co-expressed with 4F2hc, but its expression was intracellular when expressed alone in oocytes (Fig. 7).

The tissue expression of the mRNA corresponding to LAT-2 was examined by Northern blot analysis at high stringency conditions (Fig. 8). mRNA species of ~5 and ~3.7 kb hybridize with the LAT-2 cDNA; the size of the shorter transcript corresponds to that of the LAT-2 cDNA identified here. Both transcripts are expressed most conspicuously in the kidney. Placenta ≫ brain, liver > skeletal muscle, and heart also express these transcripts. The last two also showed a very faint band of ~7 kb. Long exposures also revealed the 5- and 3.7-kb transcripts in the small intestine and the lung. *In situ* hybridization studies specifically localized the renal expression of LAT-2 mRNA to the epithelial cells of proximal tubules, most probably in the convoluted part (Fig. 9). No other components of the nephron, including distal tubules and glomeruli, were reactive with LAT-2 cRNA probe. A similar pattern of expression has been shown on inmunolocalization of 4F2hc protein on kidney cortex (Ref. 28, and data not shown).

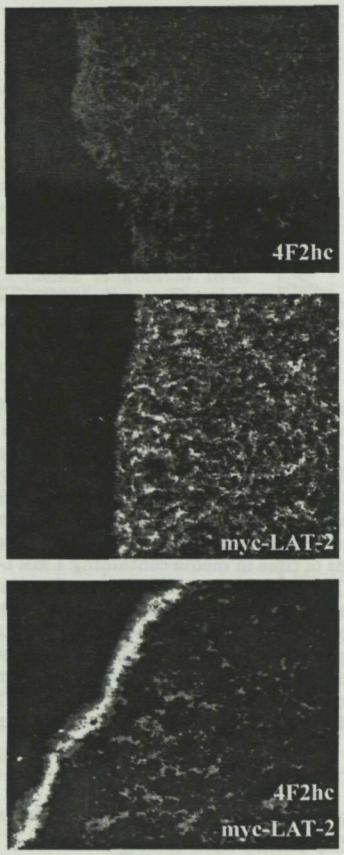

FIG. 7. **Localization of N-*myc*-LAT-2 in oocytes.** Oocytes were injected with *myc*-tagged LAT-2 or 4F2hc cRNA alone or in combination. Three days later oocytes were processed for immunocytochemistry with mAb 9E10 anti-*myc* as primary antibody and Texas red-conjugated goat anti-mouse as secondary antibody (see "Experimental Procedures"). Micrographs show that 4F2hc brings N-*myc*-LAT-2 to the oocyte plasma membrane. The *myc*-immunodetected signal (*white*) is visible inside the oocyte when expressed alone, but it is at the plasma membrane when co-expressed with 4F2hc. The signal is almost absent in 4F2hc-injected oocytes. Two days after injection the induced uptake of 100 μM L-[^{3}H]leucine in pmol/5 min per oocyte was 0.2 ± 1.0 for N-*myc*-LAT-2, 0.9 ± 0.3 for 4F2hc, 37.8 ± 3.9 for 4F2hc/LAT-2, and 19.2 ± 3.3 for 4F2hc/N-*myc*-LAT-2.

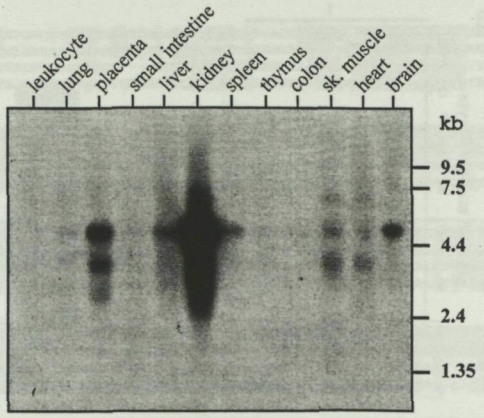

FIG. 8. **Northern blot analysis for LAT-2 mRNA in human tissues.** A poly(A) RNA membrane (2 μg per lane) containing 12 different human adult tissues was purchased from CLONTECH. Blots were probed with ^{32}P-labeled human IMAGE clone 267204 and washed at high stringency conditions (see "Experimental Procedures"). Human LAT-2 cDNA hybridizes to transcripts of ~5 and ~3.7 kb and is expressed in kidney ⋙ placenta ≫ brain, liver > spleen, skeletal muscle, heart, small intestine, and lung. A transcript of ~7 kb is also visible in skeletal muscle and heart. The leukocyte sample is from peripheral blood.

DISCUSSION

In this study we have identified a new member (LAT-2) of the family of amino acid transporters, which are subunits of 4F2hc and in humans are composed also of LAT-1, y^+LAT-1, and y^+LAT-2. We report here on the human LAT-2 cDNA sequence, chromosomal location, and pattern of expression of its mRNA. Moreover, we show that 4F2hc brings LAT-2 to the oocyte plasma membrane, which induces a system L amino acid transport activity with broad specificity. Therefore, LAT-2 is a putative new light subunit of the surface antigen 4F2.

Before the identification of the 4F2hc subunits, functional expression experiments in oocytes revealed that 4F2hc induced both system y^+L (16, 24–27) and system L (29) transport activities. In agreement with this finding, the 4F2hc subunits y^+LAT-1, y^+LAT-2, LAT-1, and LAT-2 are isoforms of systems y^+L and L, respectively. Before our cloning of system y^+L, there were no reports of variants of system y^+L in the literature (30). y^+LAT-1 is defective in lysinuric protein intolerance (5, 6), and y^+LAT-2 might be responsible for system y^+L in the cell types in which this transport activity is not defective in lysinuric protein intolerance (*e.g.* erythrocytes (31)). In contrast, system L variants L1 (substrate affinity in the micromolar range) and L2 (substrate affinity in the millimolar range) have been described previously (10). These subtypes are expressed in hepatoma cell lines and hepatocytes, respectively (11). An L3 subtype was described in fibroblasts with an affinity between that of the L1 and L2 subtypes (12). LAT-1 fits the transport characteristics and the tissue distribution of subtype L1 (Ref. 1 and present study). LAT-2 shows characteristic features of system L (*i.e.* sodium-independent transport of zwitterionic amino acids inhibitable by the analog BCH), but it also shows features that are dissimilar to both system L subtypes. Thus, LAT-2 is expressed in the liver and has a substrate affinity in the micromolar range for L-leucine (K_m ≈220 μM). This is a lower affinity than has been described for rat (18 μM) and *Xenopus* (32 μM) LAT-1 (1, 2). Moreover, in contrast to the hepatic system L, LAT-2 also transports small zwitterionic amino acids (*e.g.* L-alanine with an apparent K_m ≈ 1 mM). A transport system with similar characteristics (sodium-independent, *trans*-stimulated transport for large and small zwitterionic amino acids and a similar apparent K_m for L-alanine) to that of LAT-2 has been described in the basolateral membrane of the intestinal enterocyte (32, 33) and the placental syncytiotrophoblast (34).

What is the physiological role of a system L transporter with broad specificity for zwitterionic amino acids, including the small ones? Christensen (10) hypothesized that system L serves the exchange (efflux and influx) of zwitterionic amino acids through the plasma membrane to fulfill the inter-organ fluxes of these amino acids (10). The transport activity induced by 4F2hc/LAT-2 is highly *trans*-stimulated in oocytes. Indeed, LAT-2 behaves as an exchanger, because the efflux via this transporter in oocytes is totally dependent on the presence of a substrate in the medium. The kidney showed the highest LAT-2 mRNA expression (present study), whereas LAT-1, the other system L isoform transporter, is not expressed in the kidney (1). The involvement of *rBAT* and *y^+LAT-1* in Type I cystinuria and lysinuric protein intolerance, respectively (5, 6, 9), demonstrates the role of these transporters in the apical reabsorption of cystine and dibasic amino acids (rBAT) and in the basolateral efflux of dibasic amino acids (4F2hc/y^+LAT-1). LAT-2 mRNA is restricted to the epithelial cells of the proximal tubule of the human kidney (present study). In these cells 4F2hc has a basolateral location (28). This result suggests that

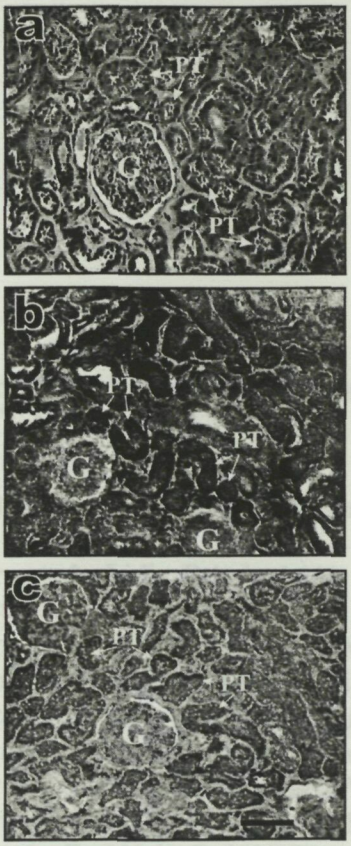

FIG. 9. ***In situ* hybridization of LAT-2 mRNA in human adult kidney.** Serial paraffin-embedded sections of human kidney cortex were stained with hematoxylin-eosin (*a*) or incubated with antisense (*b*) or sense (*c*) LAT-2 cRNA probes as described under "Experimental Procedures." Results are representative of two independent experiments. LAT-2 mRNA-specific detection is restricted to proximal tubule (*PT*) epithelial cells (some proximal tubules are indicated by *arrows*). The proximity of these tubules to the glomerulus (*G*) suggests a localization of LAT-2 mRNA signal in the proximal convoluted part. No other specific signal was detected in the renal cortex. *Bar* = 100 μm.

4F2hc/LAT-2 might mediate the efflux of zwitterionic amino acids, including those with a short side chain, through the basolateral plasma membrane. Moreover, the fact that cysteine, the main intracellular form of cystine (35, 36), strongly inhibits 4F2hc/LAT-2 transport activity suggests the role of this transporter in the renal reabsorption of cystine. The expression of LAT-2 mRNA in the small intestine suggests a similar role in the intestinal absorption of zwitterionic amino acids. The expression of LAT-2 mRNA in the placenta also implicates it in the transfer of zwitterionic amino acids from the placenta to the fetus. This hypothesis needs confirmation by the localization of LAT-2 at the basolateral plasma membrane in the epithelial cells of the proximal tubule and in the placental trophoblast. The expression of LAT-2 mRNA in the brain, liver, spleen, and skeletal muscle suggests a role in the release of glutamine and short zwitterionic amino acids from these tissues as follows: (i) glial cells release glutamine to neurons as a substrate for glutamate and γ-aminobutyric acid synthesis (37); (ii) hepatocytes in the perivenous zone release glutamine as an ammonium detoxification pathway (38); (iii) the skeletal muscle releases most of the aminic nitrogen as glutamine and alanine (39); and (iv) spleen and small intestinal enterocytes metabolize glutamine and produce alanine and other small zwitterionic amino acids (*e.g.* serine and glycine) (40). Identification of the cells expressing LAT-2 in these tissues will help us to understand the role of this system L amino acid transporter with broad specificity.

Acknowledgments— We thank Judith Garcia for technical assistance, Belen Peral for helping with and making possible the radiation hybrid analysis, and Núria Reig for help in reviewing this manuscript. We thank the Serveis Científico-Tècnics de la Universitat de Barcelona for sequencing and for help with immunochemical and *in situ* hybridization analysis. We thank the Fundació Institut d'Urologia, Nefrologia i Andrologia-Puigvert for donating human tissue and also thank Robin Rycroft for editorial help.

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Treball de col.laboració 3

Non-type I cystinuria caused by mutations in SLC7A9, encoding a subunint ($b^{0,+}AT$) of rBAT.

International Cystinuria Consortium

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Nature Genetics (1999) **23**, 52-57.

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Non-type I cystinuria caused by mutations in *SLC7A9*, encoding a subunit ($b^{0,+}$AT) of rBAT

International Cystinuria Consortium

Cystinuria (MIM 220100) is a common recessive disorder of renal reabsorption of cystine and dibasic amino acids. Mutations in *SLC3A1*, encoding rBAT, cause cystinuria type I (ref. 1), but not other types of cystinuria (ref. 2). A gene whose mutation causes non-type I cystinuria has been mapped by linkage analysis to 19q12–13.1 (refs 3,4). We have identified a new transcript, encoding a protein ($b^{0,+}$AT, for $b^{0,+}$ amino acid transporter) belonging to a family of light subunits of amino acid transporters, expressed in kidney, liver, small intestine and placenta, and localized its gene (*SLC7A9*) to the non-type I cystinuria 19q locus. Co-transfection of $b^{0,+}$AT and rBAT brings the latter to the plasma membrane, and results in the uptake of L-arginine in COS cells. We have found *SLC7A9* mutations in Libyan-Jews, North American, Italian and Spanish non-type I cystinuria patients. The Libyan Jewish patients are homozygous for a founder missense mutation (V170M) that abolishes $b^{0,+}$AT amino-acid uptake activity when co-transfected with rBAT in COS cells. We identified four missense mutations (G105R, A182T, G195R and G295R) and two frameshift (520insT and 596delTG) mutations in other patients. Our data establish that mutations in *SLC7A9* cause non-type I cystinuria, and suggest that $b^{0,+}$AT is the light subunit of rBAT.

We identified a human kidney cDNA, *SLC7A9*, which encodes a new member of a family of amino acid transporters[5–10]. *SLC7A9* cDNA is 1,814-bp, polyadenylated and has an ORF encoding 487 amino acid residues (data not shown). Multialignment of the protein $b^{0,+}$AT with the other human members of the family is shown (Fig. 1). Northern-blot analysis showed that $b^{0,+}$AT is expressed in kidney, liver, small intestine and placenta as a transcript of approximately 1.9 kb, the size of *SLC7A9* cDNA (data not shown). This tissue distribution is consistent with that of a tentative rBAT light subunit[11].

As expected for an rBAT subunit, $b^{0,+}$AT brings rBAT to the plasma membrane in co-transfected COS cells (Fig. 2). In contrast, transfection of rBAT alone resulted in the blockage of the expressed protein in the endoplasmic reticulum (ER; Fig. 2), as reported[12]. Similar behaviour was found for 4F2hc and its subunits when co-injected in *Xenopus laevis* oocytes[6,8,9,13]. Co-injection of rBAT or 4F2hc with $b^{0,+}$AT cRNAs in oocytes does not induce expression of amino acid transport (data not shown). In the case of rBAT this is probably due to the high amino acid transport induced by rBAT alone in oocytes. In contrast, co-transfection of rBAT and $b^{0,+}$AT increased sodium-independent uptake of L-arginine in COS cells (Fig. 3).

YAC library and BAC contig screening indicated that *SLC7A9* maps to YAC 877f9 and BAC356357 (data not shown). This BAC localized between microsatellite marker loci *D19S776* and *D19S786* (ref. 14), in the refined locus of non-type I cystinuria. Thus, analysis of recombinant events in Spanish and Italian families, and historical recombinant events in Libyan Jewish families, including new marker loci[15] (*C17A* and *C17* in cosmid R32329, *B27A* in BAC 277469 and *B35* in BAC35635), confined the gene to a 1.3-Mb interval between *C17A* and *D19S874* (centromere...*C17A/D19S225/C17-D19S868-D19S776-B27A/B35-D19S786-D19S416-D19S213-D19S874*...telomere; refs 15, 16 and data not shown). This data suggests *SLC7A9* as a candidate gene for non-type I cystinuria.

We searched for sequence alterations in *SLC7A9* in non-type I cystinuria Libyan Jewish and Italian patients, and in unclassified cystinuria patients from North America, with no mutations in the coding region of *SLC3A1*. We also screened patients with mixed type I/non-type I cystinuria from Italy. Sequencing of the entire cDNA in 3 Libyan-Jewish homozygotes revealed a c693G→A substitution, resulting in a mutation at position 170 of a fully conserved valine to methionine (Figs 1 and 4). Restriction analysis showed that the V170M mutation was found in 16 of 17 independent carrier chromosomes of 23 patients analysed (Table 1), but not in 200 control chromosomes. Restriction analysis also showed mendelian inheritance of V170M in all patients (data not shown). In one carrier chromosome from

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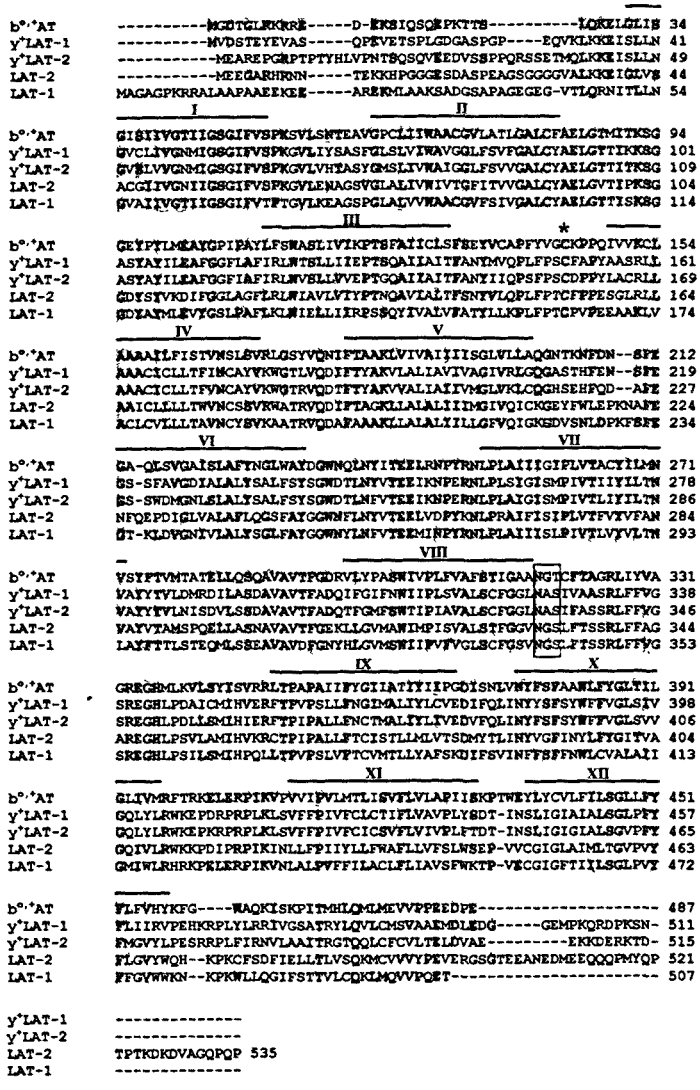

Fig. 1 Amino acid sequence comparison of the five human members of the family of light subunits of heteromeric amino acid transporters. Multialignment was done using the program CLUSTALW Sequence Alignment from the Baylor College of Medicine. $b^{0,+}$AT has an amino acid sequence identity of 40%, 40%, 42% and 39% with human y^+LAT-1, y^+LAT-2, LAT-1 and LAT-2, respectively. The horizontal lines indicate the 12 putative transmembrane domains determined by computer analysis. The amino acid residues identical to $b^{0,+}$AT sequence are indicated in grey boxes. The solid frame box indicates a potential N-glycosylation site, conserved in all members of this family of amino acid transporters, but according to our membrane topology prediction, this site is intracellular and cannot be glycosylated. The conserved cysteine residues, which are shown to form the disulfide bridge between 4F2hc (residue C109 in the human sequence) and the *Xenopus laevis* LAT-1 (residue C164) subunit[29], are indicated with an asterisk (cysteine residue 144 in $b^{0,+}$AT). Mouse xCT, amino acid transporter and subunit of 4F2hc of this family of proteins[10], is not shown in this multialignment because its human counterpart has not been cloned.

Sequence analysis of 3 exons, covering 30% of the coding region of *SLC7A9*, revealed 6 mutations in North American and Italian patients between transmembrane domains III and VII (Table 1 and Fig. 4): 4 missense mutations (G105R, A182T, G195R and G259R) and 2 frameshift mutations (520insT and 596delTG), leading to premature stop codons 24 and 208 bp downstream, respectively. G105R is the most common mutation, with 3 homozygous, 3 homo- or hemizygous and 11 heterozygous patients detected (Table 1). G259R was detected in homozygosity in a daughter of a consanguineous mating (Fig. 5 and Table 1), whereas mutation 596delTG was found in a homo- or hemizygous state in a North American patient (Table 1). A182T was found in 4 unrelated Spanish patients (Table 1). For the Italian and Spanish patients, restriction analysis showed mendelian inheritence of these mutations in all cases. None of these six mutations were identified in 100–130 control chromosomes.

Of the seven non-type I cystinuria-specific mutations described, two are frameshift mutations leading to truncated $b^{0,+}$AT proteins that lack the last three-quarters of the protein, and three others (G105R, V170M and G195R) are missense mutations of highly conserved amino acid residues (Figs 1 and 4). To demonstrate that one of these missense mutations, V170M (the Libyan Jewish mutation), causes cystinuria, we analysed sodium-independent uptake of L-arginine after transient co-transfection of rBAT with constructs encoding wild-type $b^{0,+}$AT or V170M $b^{0,+}$AT in COS cells. The V170M substitution abolished the L-arginine uptake associated with the co-expression of rBAT (Fig. 3). These results demonstrate that mutations in *SLC7A9* cause non-type I cystinuria.

individual 176-02 (ref. 3), we did not find any mutation despite sequencing the entire coding region. Urine amino acid levels in this individual were consistent with a non-type I carrier, and this family showed linkage to chromosome 19q (ref. 3). We suspect that this chromosome bears a mutation in the non-coding region of *SLC7A9*. This is supported by the fact that *SLC7A9* lies in BAC356357 together with marker *B35*, and this chromosome has a different allele from the ancestral one in this and neighbouring markers (data not shown).

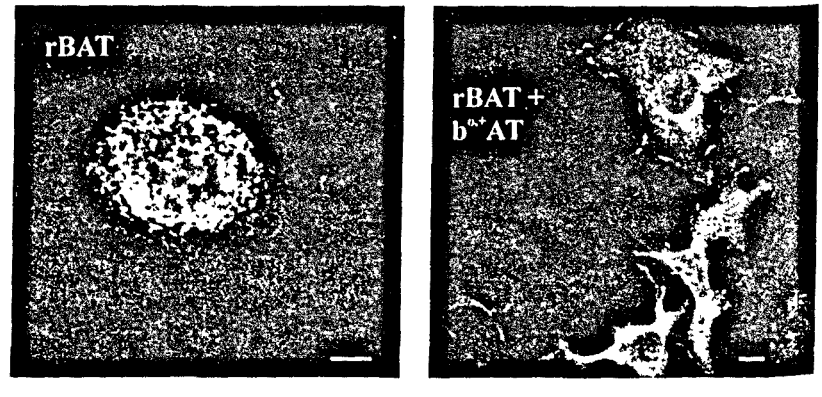

Fig. 2 Localization of rBAT in transfected COS cells. COS-1 cells were transfected with pcDNA3-rBAT, pcDNA3-rBAT and pcDNA3-$b^{0,+}$AT or with the vector alone (pcDNA3). After 24 h, rBAT was immunolocalized with anti-rBAT MANR5 polyclonal antibody[19]. The rBAT-immunodetected signal (white) is almost exclusively visible inside the cell, most probably in the ER and lysosomes, in rBAT-transfected cells. In contrast, in ~10% of the cells transfected with constructs encoding rBAT and $b^{0,+}$AT the rBAT-specific signal reached the plasma membrane. Background in vector-transfected cells was undetectable (data not shown). Bars, 10 μm.

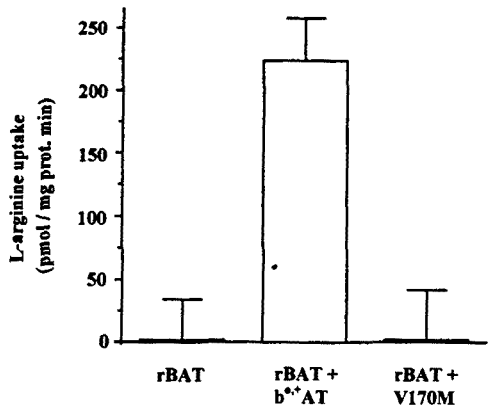

Fig. 3 Analysis of V170M $b^{0,+}$AT amino acid uptake in COS cells. COS-1 cells were transfected with pcDNA3-rBAT, pcDNA3-rBAT and pcDNA3-$b^{0,+}$AT or vector alone (pcDNA3). The uptake of 50 μM L-[^{3}H] arginine in the absence of sodium was measured 48 h after transfections. The specific uptake activity (that is, subtracting the uptake in vector-transfected cells) is shown. Data are mean±s.e.m. from four determinations. The specific uptake in the cells transfected with rBAT and $b^{0,+}$AT was significantly higher (Student *t*-test; $P \leq 0.01$) than that of rBAT-transfected cells or rBAT- and V170M-transfected cells. The uptake in vector-transfected cells was 590±20 pmol L-arginine/mg protein/min.

We were not able to fully differentiate between the type II and III phenotypes. According to the urinary amino acid profile, most of the patients described above seemed to have inherited type III cystinuria from both parents, but there are some exceptions (Table 1). V.S. carries mutation G105R and appears to be a type II/II cystinuria patient, on the basis of the urine excretion of his parents. This family alone yielded a lod score greater than 2 using markers close to the cystinuria 19q locus[4]. On the other hand, mutation G105R segregates with type III in 10 Italian families. Similarly, mutation V170M in Libyan-Jewish patients associates with type II or III urinary phenotypes[17]. Moreover, some mutations in *SLC7A9* (for example, A182T) result in a very mild phenotype similar to type I or mild type III phenotypes, whereas others (for example, G105R and G195R) result in a type III phenotype in some patients who inherited mixed-type cystinuria. These results suggest that types II and III, and in some cases type I, represent allelic differences in *SLC7A9*. Other factors, genetic and environmental, are probably also involved. Finally, mutations G105R in *SLC7A9* and M467T in *SLC3A1* (data not shown) were found in one patient (N.G.). These preliminary results suggest that cystinuria is a digenic disease in some of the mixed type I/non-type I patients, and support the hypothesis of partial genetic complementation[18]. Full sequencing of the two *SLC7A9* alleles in this family will be necessary to confirm this.

Studies that we have performed in patients with mutations in *SLC7A9* and with type III/III cystinuria based on the urinary amino acid profile of their parents show an impairment (~50% of control values) of intestinal absorption of lysine and arginine (ref. 17, and L.d.S. and A.P., unpublished data). This is in agreement with the expression of $b^{0,+}$AT in the small intestine.

Localization[19,20] and partial knockout studies[21] showed that rBAT is necessary for the activity of the amino acid transport $b^{0,+}$ in the brush-border plasma membrane of epithelial cells in the proximal straight tubule. The functional studies of $b^{0,+}$AT and rBAT described here, and the fact that mutations in their genes cause non-type I and type I cystinuria, respectively, support the hypothesis that $b^{0,+}$AT and rBAT are the light and heavy subunits, respectively, of the amino acid transporter $b^{0,+}$. Consistent with this, it was shown that rBAT and $b^{0,+}$AT form a disulfide-linked heterodimer, and that a fusion of the two proteins expresses $b^{0,+}$ transport activity in oocytes (F. Verrey *et al.*, pers. comm.).

It remains to be determined why mutations in *SLC3A1* are recessive, whereas mutations in *SLC7A9* are incompletely recessive. We offer two hypotheses. First, if the active $b^{0,+}$ transporter is constituted by more than one rBAT and $b^{0,+}$AT subunits, one mutated allele of $b^{0,+}$AT might produce a dominant defect, whereas one mutated allele of rBAT would produce a trafficking defect, as previously described[22]. Second, the light subunit of rBAT ($b^{0,+}$AT) might associate with a protein other than rBAT, and express cystine transport activity in a different proximal tubular segment. *In situ* hybridization and immunolocalization studies (F. Verrey *et al.*, pers. comm.) show expression of $b^{0,+}$AT in the epithelial cells of the proximal straight tubule, like rBAT, but higher expression in the proximal convoluted tubule. Most of the renal cystine reabsorption occurs in the proximal convoluted tubule via a low-affinity system not identified at the molecular level[23]. If *SLC7A9* also encodes this transport system, a partial

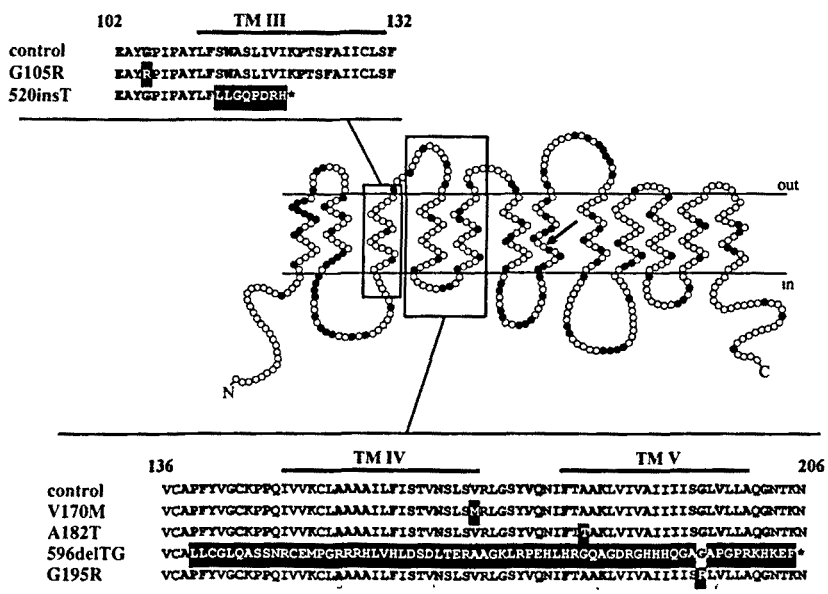

Fig. 4 Schematic representation of the non-type I cystinuria-specific mutations identified in the $b^{0,+}$AT amino acid transporter. Membrane topology prediction algorithms suggest that $b^{0,+}$AT contains 12 transmembrane domains with the N and C termini located intracellularly. All mutations have been identified in the part of the protein between transmembrane (TM) III and VII. The missense mutations G105R, V170M (The Libyan Jewish mutation) and G195R change residues that are highly conserved in this family of amino acid transporters. The arrow in TMVII denotes the amino acid residue altered in mutation G259R. Control, wild-type sequence. *, premature stop codon. Grey background denotes amino acid residues conserved in the human members of the family. White characters on black background denote sequence alterations versus wild-type sequence. TM, putative transmembrane domain. Amino acid residue numbers are indicated above the alignments.

Table 1 • Non-type I cystinuria-specific mutations in *SLC7A9*

Mutation	Nucleotide change	Restriction analysis creates	Restriction analysis destroys	Patients	Type of cystinuria	Mutation genetic status
G105R	c496G→A	–	*Apa*I	brothers T G and T G (USA)	unknown	homo- or hemizygous[a]
				M S (USA)	unknown	homo- or hemizygous[a]
				F G , R W and J P (USA)	unknown	heterozygous
				V R , M G and L.F (Italy)	non-type I	homozygous
				R L , B L , A E , C S , B F , R S and V S [b] (Italy)	non-type I	heterozygous
				N G (Italy)	non-type I[c]	heterozygous
520insT[d]	ins T at c520	–	–	G W (USA)	unknown	heterozygous
V170M	c693G→A	–	*Rsa*I[e]	23 Libyan Jewish patients[f] (16 independent chromosomes)	non-type I	homozygous in 20 patients heterozygous in 3 patients
A182T	c729G→A	–	*Ksp*I	P M (Italy)	non-type I[g]	heterozygous
				M C (Spain)	non-type I[h]	heterozygous
				M R , E B and R P (Spain)	non-type I[g]	heterozygous
596delTG[d]	del TG at c596	–	–	T C (USA)	unknown	homo- or hemizygous[a]
G195R	c768G→A	–	*Acl*I	C M (Italy)	non-type I[b]	heterozygous
G259R	c960G→A	*Dde*I[e]	–	M F (Italy)	non-type I	homozygous

All are unrelated patients, unless otherwise indicated [a]Mendelian inheritance was confirmed in all cases, except for these North American patients for whom parent samples were not available Non type I patients are type III, except [b]V S (type II/II patient) and the [f]Libyan Jewish patients, who showed a urinary phenotype range between type III and type II [c]Mutation transmitted as the non type I allele in mixed type I/non type I cystinuria [d]These mutations were checked by sequencing in normal chromosomes [e]Restriction site generated by a mutagenesis primer [g]Mutation transmitted as the type I or a mild non type I allele in mixed type I/non type I cystinuria [h]Doubtful non type I or mixed type I/non type I cystinuria The phenotype of Libyan Jewish patients and patients B L (family F3), P M (F25), B F (F40), C S (F41), A E (F43), R S (F48), V S (F62) and M R (F15) have been reported[2,4,17]

defect in this major renal reabsorption mechanism would explain the incompletely recessive phenotype of non-type I cystinuria

We have explained 41 cystinuria chromosomes of 133 independent chromosomes In most patients, however, we have sequenced only one-third of the *SLC7A9* coding region. In the remaining 92 chromosomes, mutations may occur in *SLC7A9* regions which have not yet been sequenced, or in other genes. An additional member of the solute carrier family 7 is located, like *SLC7A9*, in the non-type I cystinuria locus and remains to be studied. Full mutational analysis of *SLC7A9* and this new putative amino acid transporter might clarify the subtypes of non-type I cystinuria, and the molecular basis of the disease.

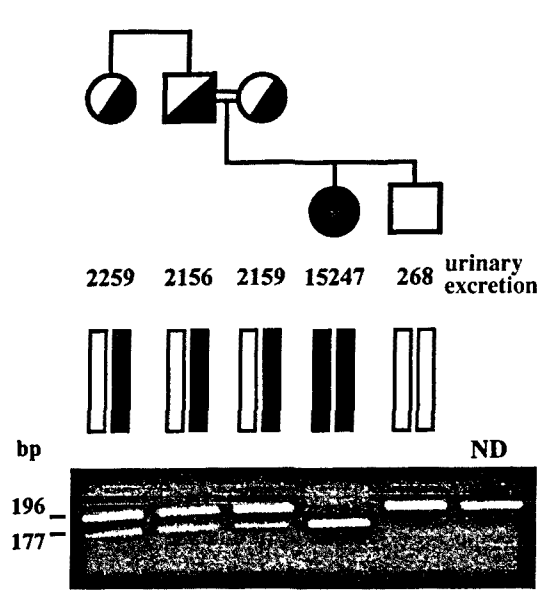

Fig. 5 Mendelian inheritance of mutation G259R of *SLC7A9* in a non-type I cystinuria consanguineous family Haplotypes (vertical open and filled bars) were obtained with markers from cystinuria non type I locus on chromosome 19 (*D19S75, D19S251, D19S213, D19S416* and *D19S208*) Open chromosomes are normal, but not necessarily identical, and filled chromosomes are identical cystinuria-transmitting chromosomes The sum of the urine excretion of cystine, lysine, arginine and ornithine is reported for each individual as μmol/g creatinine Detection of mutation G259R by the *Dde*I site generated using BR1 and the forward mutagenesis primer BF2 (5 –CTGCCTTTGGCCATTATCCTC–3 , underlined character indicates the mutated nucleotide) is shown The non digested (ND) band is 196 bp, which results in two bands of 177 bp and 19 bp (not shown) after digesting the mutated allele with *Dde*I

Methods

Cystinuria patients We studied 23 non-type I cystinuria Libyan Jewish patients, 33 unclassified cystinuria patients from North America and 27 non-type I or mixed type I/non-type I cystinuria patients from Italy, representing 17, 62 and 54 independent chromosomes, respectively All patients excrete cystine and dibasic amino acids in the range of cystinuria homozygotes[2], and all but one North American patient and three Libyan Jewish patients have documented production of cystine calculi (refs 2,4,17, and data not shown) Cystinuria in these Libyan Jewish patients was classified as a non-type I disease in a previous study[17] Despite the genetic homogeneity of these Libyan Jewish patients, suggesting a common founder mutation, the range of amino acid urinary excretion in obligate heterozygotes is large enough for some individuals to be classified as type II and

some as type III patients[17]. The North American patients have not been classified because samples from their parents are not available, but they do not show mutations in the ORF of *SLC3A1* after a mutation search by SSCP. The obligate heterozygotes of the families of the Italian patients, and the four Spanish patients, showed urine phenotypes in the non-type I or mixed type I/non-type I cystinuria ranges[2]. Urine phenotype of the non-type I cystinuria in these patients corresponds to the type III range (refs 2,18). Only the Italian patient V.S. inherited cystinuria from parents excreting cystine and dibasic amino acids over the type III range[2], who were classified as type II heterozygotes[4].

EST database searching. We performed dbEST (ref. 24) searches as described[7,25]. We obtained the cDNA clone corresponding to a partial $b^{0,+}$AT transcript (IMAGE clone 126710) from Research Genetics and the UK Human Genome Mapping Project (HGMP) Resource Centre.

cDNA library screening. Phage library plating and screening conditions were as described[26]. Human cDNA clones were isolated from an adult human kidney cDNA library (Stratagene; λ Uni-ZAP XR vector) using a 742-bp 126710 probe, labelled with [α-^{32}P]dCTP, obtained using primers P6D6 (5′–CTGC-CTTTGGCCATTATCATCGGGA–3′) and P6R4 (5′–CAGCTGACTTGGC-TACAAGAGA–3′), designed from the EST clone 126710. Nine positive plaques, confirmed by nested-PCR amplification, were excised according to the manufacturer's instructions. One clone was purified and sequenced.

DNA sequencing and computer sequence analysis. We carried out automated sequencing (using Applied Biosystems ABI 377 fluorescent sequencers) using gene-specific oligonucleotide primers. Sequences of these primers are available on request. Sequence assembly and editing was performed using Sequencher (Gene Codes), AutoAssembler (Perkin Elmer-Applied Biosystems) and DNA Strider 1.2 software programs and multiple sequence alignment was performed using ClustalW algorithm, as described[7,25]. We compared nucleotide and amino acid sequences with the non-redundant sequence databases at the NCBI (National Center for Biotechnology Information) using BLAST version 2.0 as described[25]. The prediction of transmembrane domains was performed as described[7].

Northern-blot analysis. We used a human adult poly(A$^+$) RNA membrane containing 12 different tissues (Clontech). The same 742-bp $b^{0,+}$AT PCR-amplified fragment used for the library screening was used as a probe. Hybridization and washing were as recommended by the manufacturer.

Cell culture, transfection, amino acid uptake and immunofluorescence in COS cells. COS-1 cells (a gift from S. Vilaró) were cultured in DMEM supplemented with 10% fetal bovine serum, antibiotics and glutamine (2 mM). For amino acid uptake measurements, cells were cultured in six multi-well dishes. For immunofluorescence studies, cells were cultured in 10-cm diameter plates with glass coverslips.

For transfection studies, expression vectors pcDNA3, pcDNA3-human rBAT (cloned between the restriction sites *Eco*RI-*Xba*I), pcDNA3-human $b^{0,+}$AT and pcDNA3-human V170M $b^{0,+}$AT (cloned between the restriction sites *Eco*RI-*Xho*I) were used. At ~60% confluence, we transiently transfected cells with DNA (2 μg) using the FuGENE 6 transfection reagent (Boehringer) following the manufacter's protocol. After 48 h, uptake of L-[^{3}H] arginine (50 μM) was measured as described[11]. Results of uptake are corrected for protein content, and expressed after subtracting the background values.

For immunofluorescence labelling, the transfected COS-1 cells grown on glass coverslips were rinsed briefly in PBS, fixed in 3% paraformaldehyde phosphate buffer saline (PBS) for 30 min at RT, washed twice in PBS and processed. We used anti-rBAT MANR5 (1:500) as primary antibody[19]. Primary antibody was visualized with Oregon green-conjugated goat anti-rabbit IgG (Molecular Probes) at a 1:100 dilution. Both antibodies were diluted in PBS containing 1% FBS. Finally, coverslips were mounted with immunofluorescence medium (ICN). Confocal microscopy was performed at the Serveis Científico-Tècnics of the University of Barcelona.

RT-PCR amplification and mutation analysis of *SLC7A9* cDNA. For the identification of V170M mutation, RNA from lymphoblastoid cells from three Libyan Jewish patients and a control was isolated, retrotranscribed with primer P6R7 (5′–CTGAGTATATTTTATTCGTAAGA–3′) and PCR-amplified with primers P6F7 (5′–CAGGAGGAAACATGGGGGATACTG –3′) and P6R3 (5′–CTGCAGGAGTTCGGTGGCA–3′). We performed a second PCR with primers P6D11 (5′–GGTCATCTCGACAGTGAACT–3′) and P6R1 (5′–CACGTTGATGAGGATGTAGCA–3′). The resulting fragment was sequenced from both sides with primers P6D11 and P6R1. RT-PCR protocols were as described[27].

PCR-amplification and mutation analysis of genomic *SLC7A9*. For the screening of V170M mutation, an 84-bp genomic DNA was PCR-amplified with *SLC7A9* forward primer 5′–CATCTTGTTCATCTCGACAGTGAA CTC–3′ and with *SLC7A9* reverse mutagenesis primer 5′–CCA GCTTGGCCGCGGTGAAGATGTTCTGGACGTAGCTTCCCAGCCGTA–3′ (the underlined base has been changed to create an *Rsa*I site in the wild-type *SLC7A9* sequence, but not in V170M *SLC7A9* sequence). This restriction site does not depend on the V170M-associated c692 T→C polymorphism (nt 5′ next to the mutation).

Lymphoblastoid cell lines were not available for any of the North American or for most of the Italian patients studied, therefore mutational screening in these patients was performed with genomic DNA. We screened a PAC library by hybridization in standard conditions, using as a probe a 675-bp cDNA fragment encompassing both the 3′ UTR and part of the coding region (nt positions 1,000–1,675 of the cDNA). Four PAC clones from the RPIC5 library were identified (10003N9, 911F19, 989n8 and 852F21). Sequences of exon-intron boundaries in these clones allowed the design of the oligonucleotide primers used to amplify genomic DNA (G105R, 596delTG and 520insT, DF, 5′–AGCCTCCGGTGGGAGG AAG–3′, and DR, 5′–GAGTCCCCAGACACCCTCTG–3′; A182T and G195R, LAT3EF, 5′–AAAGGAGACTCTCTCCAGGG–3′, and RevA, 5′–ATGCTTCCTTGGAGATGGGCT–3′; and G259R, BF, 5′–CTGAACGTG GGTCTCCGTG–3′, and BR1, 5′–ACCTCCAGTGCTGACACCTG–3′). These primers amplify three exons of *SLC7A9* covering nt 421–663, 664–789 and 935–1,058 of the cDNA (the first ATG starts at nt 186). Patient DNA (100 ng) was amplified with intron-derived oligonucleotide primers for each exon analysed, using the following PCR conditions (total volume of 50 μl): dNTPs (200 μM of each), PCR buffer containing $MgCl_2$ (1.5 mM, final concentration), Taq Gold Polymerase (0.02 units/μl, Perkin-Elmer) and primers (0.35 μM of each). The cycling conditions were set according to the Taq Gold manufacturer's instructions; the annealing temperature was 57 °C for all sets of primers. In the search for *SLC7A9* mutations in North American patients, we sequenced amplified fragments with the same primers used for PCR amplification. For the search of mutations in Italian patients, genomic DNA was analysed by RNA-SSCP technology as described[28]. After PCR reaction with primers DF and DR, LAT3EF and RevA, and BF and BR1 (the forward primers DF, LAT3EF and BF containing a 5′ end tail with the T7 promoter sequence), transcription was carried out with T7 RNA polymerase (10 U) in a final volume (10 μl) containing DTT (10 mM), Tris (40 mM, pH 7.5), $MgCl_2$ (6 mM), spermidine (2 mM), NaCl (10 mM), ribonucleotides (5 nmol each), Rnasin (10 U) and of S^{35}-UTP (0.2 μl, >1,000 μCi/μmol). We mixed transcribed RNA (2 μl) with 95% formamide (48 μl), EDTA (20 mM), 0.05% bromophenol blue and 0.05% xylene cyanol. The mixture was heated to 95 °C for 6 min and then chilled on ice for 10 min. An aliquot of 4.4 μl was then loaded onto a 6.5% non-denaturing polyacrylamide gel. After electrophoresis (18 W constant power for 16 h), the gel was dried and subjected to autoradiography for 12 h. Bands showing an electrophoretically altered mobility were then sequenced with the same primers used for PCR amplification.

Construction of V170M $b^{0,+}$AT cDNA. For the construction of the V170M-human $b^{0,+}$AT we used the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. The mutagenic oligonucleotide was 5′–GAACTCACTGAGC(A)TGCGGCTGGGAAG–3′ (sense strand, the mutated nt 693 is indicated by parentheses). Proper construction of the mutated cDNA was confirmed by complete sequencing.

Development of three new microsatellite markers in the non-type I cystinuria locus. We started with a contig of cosmids (provided by L. Ashworth) and chromosome 19q13.1 BACs (Research Genetics). All are physically mapped in available restriction maps (http://www-bio.llnl.gov/rmap), which are anchored to the metric map of chromosome 19

(ref 14; available updated at http://www-bio.llnl.gov/genome-bin/loadmap?region=mq). A Southern blot containing *Eco*RI cosmid digests was probed with an $(AC)_{10}$ oligonucleotide. A positive 700-bp fragment from cosmid R32329 was subcloned into pBS (KS, Stratagene) and sequenced with vector primers to give *C17A* marker. The clones that provided *B27A* and *B35* were selected after shotgun subcloning in pBS of BAC277469 (BAC 331g19, Research Genetics Library 193) and BAC356357 (BAC 536n17, Research Genetics Library 193), respectively, and hybridization of the subclones with an $(AC)_{10}$ oligonucleotide. *B27A* was sequenced with vector primers. As the clone for *B35* was larger, primers flanking the repeat sequence were identified after sequencing out the repeat. The primers used to amplify the three markers were: 5′–TTTTGCCTGATGTGGACCTA–3′ and 5′–TAGGTGGTGAATCATGGCTG–3′ for *C17A*; 5′–GGTAGATGTTGCCTTGTCC–3′ and 5′–CAAGATTGCACCACTACATTCC–3′ for *B27A*, 5′–TGGTGTTCAGCAAAATAAGTG–3′ and 5′–CCACAAACTTTGATGGTGA–3′ for *B35*. Information about allele frequencies, heterozygosity, assay conditions, and so on is available at the NCBI dbSNP.

GenBank accession numbers. *SLC7A9*, AF141289; microsatellite markers *B27A*, *B35* and *C17A*, AF139861, AF139862 and AF139863, respectively.

Acknowledgements

We thank the families for contributing, A Ros for cell culture; J García for technical assistance, the YAC Screening Center at San Raffaele Biomedical Science Park and the Serveis Científico-Tècnics de la Universitat de Barcelona for some of the sequences; and R Rycroft for editorial help. This research was supported in part by Dirección General de Investigacion Científica y Técnica research grant PM96/0060 from Spain to M P; BIOMED2 CT98-BMH4-3514 EC grant to V N, M P and P.G and CT97-BMH4-2284 (EURO-IMAGE) EC grant to X E. and A B, Fundació La Marató-TV3 research grant 981930 to V N and M P; Generalitat de Catalunya Grants 1997 SGR 121 and 1997 SGR 00085, Italian Telethon Foundation grants (E556) to L Z; the Merck Genome Research Institute (MGRI grant 37); and Ministero Italiano della Sanità grant to P G. E F and D T are recipients of fellowships from the Ministerio de Educación y Cultura (Spain), M P, L F, J P, M F, N R and R E are recipients of fellowships from the Comissió Interdepartamental de Recerca i Innovació Tecnologica (Catalonia, Spain), and J K.E was supported by a research fellowship from The National Kidney Foundation

Received 12 April, accepted 15 July 1999

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Treball de revisió 1

The molecular basis of cystinuria: the role of the rBAT gene

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Amino Acids (1996) **11**, 225-246.

El doctorand va participar en la correcció i revisió de l'article.

11: 207-228, 1996

Amino Acids •: ••–••

Amino Acids
© Springer-Verlag 1996
Printed in Austria

The molecular basis of cystinuria: the role of the rBAT gene

Review Article

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Accepted February 26, 1996

Summary. The cDNAs of mammalian amino acid transporters already identified could be grouped into four families. One of these protein families is composed of the protein rBAT and the heavy chain of the cell surface antigen 4F2 (4F2hc). The cRNAs of rBAT and 4F2hc induce amino acid transport activity via systems $b^{0,+}$ -like and y^+L -like in *Xenopus* oocytes respectively. Surprisingly, neither rBAT nor 4F2hc is very hydrophobic, and they seem to be unable to form a pore in the plasma membrane. This prompted the hypothesis that rBAT and 4F2hc are subunits or modulators of the corresponding amino acid transporters. The association of rBAT with a light subunit of ~40 kDa has been suggested, and such an association has been demonstrated for 4F2hc.

The $b^{0,+}$-like system expressed in oocytes by rBAT cRNA transports L-cystine, L-dibasic and L-neutral amino acids with high-affinity. This transport system shows exchange of amino acids through the plasma membrane of *Xenopus* oocytes, suggesting a tertiary active transport mechanism. The rBAT gene is mainly expressed in the outer stripe of the outer medulla of the kidney and in the mucosa of the small intestine. The protein localizes to the microvilli of the proximal straight tubules (S3 segment) of the nephron and the mucosa of the small intestine. All this suggested the participation of rBAT in a high-affinity reabsorption system of cystine and dibasic amino acids in kidney and intestine, and indicated rBAT (named SLC3A1 in Gene Data Bank) as a good candidate gene for cystinuria. This is an inherited aminoaciduria due to defective renal and intestinal reabsorption of cystine and dibasic amino acids. The poor solubility of cystine causes the formation of renal cystine calculi. Mutational analysis of the rBAT gene of patients

with cystinuria is revealing a growing number (~20) of cystinuria-specific mutations, including missense, nonsense, deletions and insertions. Mutations M467T (substitution of methionine 467 residue for threonine) and R270X (stop codon at arginine residue 270) represent approximately half of the cystinuric chromosomes where mutations have been found. Mutation M467T reduces transport activity of rBAT in oocytes. All this demonstrates that mutations in the rBAT gene cause cystinuria.

Three types of cystinuria (types, I, II and III) have been described on the basis of the genetic, biochemical and clinical manifestations of the disease. Type I cystinuria has a complete recessive inheritance; type I heterozygotes are totally silent. In contrast, type II and III heterozygotes show, respectively, high or moderate hyperaminoaciduria of cystine and dibasic amino acids. Type III homozygotes show moderate, if any, alteration of intestinal absorption of cystine and dibasic amino acids; type II homozygotes clearly show defective intestinal absorption of these amino acids. To date, all the rBAT cystinuria-specific mutations we have found are associated with type I cystinuria (~70% of the chromosomes studied) but not to types II or III. This strongly suggests genetic heterogeneity for cystinuria. Genetic linkage analysis with markers of the genomic region of rBAT in chromosome 2 (G band 2p16.3) and intragenic markers of rBAT have demonstrated genetic heterogeneity for cystinuria; the rBAT gene is linked to type I cystinuria, but not to type III. Biochemical, genetic and clinical studies are needed to identify the additional cystinuria genes; a low-affinity cystine reabsorption system and the putative light subunit of rBAT are additional candidate genes for cystinuria.

Keywords: ••

Introduction

The transport of amino acids through the plasma membrane of mammalian cells is mediated by proteins that recognize, bind and translocate these metabolites between the intra and the extracellular compartments. In the last five years the molecular biology strategies applied to amino acid transport in mammalian cells have transformed the field; recent technology based upon concepts from biochemistry, cellular biology, genetics and medicine are now available to the study of amino acid transport in mammalian cells. A growing number of mammalian cDNA sequences coding for proteins related to plasma membrane amino acid transport are known (approximately 20 cDNAs without taking into account species counterparts; see recent reviews Bertran et al., 1994; Kanai et al., 1994; Kanner and Kleinberger-Doron, 1994; Macleod et al., 1994; MacGivan and Pastor-Anglada, 1994). Besides the list is not yet complete; physiologically significant amino acid transport activities like the sodium-dependent systems A, NBB, $B^{0,+}$ and N, as well as sodium-independent systems like the ubiquitous system L and those for anionic amino acids have not yet been cloned. The mammalian amino acid transporters that have been identified, can be grouped into four gene families: 1) sodium-

independent transporters for cationic amino acids; CAT isoforms, 2) amino acid transporters for GABA, b-amino acids, taurine, glycine and proline, in the superfamily of sodium- and chloride-dependent neurotransmitter transporters, 3) sodium- and potassium-dependent transporters for anionic and zwitterionic amino acids (isoforms of system X^-_{AG} for glutamate and system ASC). The proteins deduced from the cDNA sequences, or in a few cases, the study of the purified proteins, revealed as a common structural characteristic a hydrophobic nature with the presence of 8 to 12 putative transmembrane domains. 4) In contrast to these transporters, two homologous proteins, rBAT (also named D2, NAA-Tr, NBAATr or NABT) and the heavy chain of the surface antigen 4F2 (4F2hc), are less hydrophobic and contain, depending on the structure model, 1 to 4 transmembrane domains, but induce amino acid transport activity via system $b^{0,+}$-like and y^+L-like in *Xenopus* oocytes, respectively. The apparent inability of these proteins to form a pore through the plasma membrane, due to their low hydrophobicity, prompted the hypothesis that they may be modulators of transporters with a heteromeric structure. These two proteins are the subject of the present review. Recently, it has been demonstrated that mutations in rBAT cause classic cystinuria, an inherited defect of renal and intestinal reabsorption of cystine and dibasic amino acids. The role of rBAT in cystinuria is helping us to understand the molecular basis of this disease, known from the first description of cystine calculi by Wollaston (1810), during the lifetime of Beethoven in Vienna, and described later as one of the "inborn errors of metabolism" by Sir Archibald E. Garrod (1908).

Cloning and identification of rBAT and 4F2hc as a new family of proteins involved in amino acid transport

Amino acid transport expression in *Xenopus* oocytes was used independently in three labs to clone cDNAs of a putative transporter from rabbit, rat and human kidney; homology between these proteins is very high (~85% identity) (Bertran et al., 1992c, 1993; Lee et al., 1993; Tate et al., 1992; Wells and Hediger, 1992). For clarity the name rBAT will be used for all these cDNAs and proteins in this review. The deduced rBAT protein amino acid sequence has 30% identity (~50% similarity) with the heavy chain of the cell surface antigen 4F2 (4F2hc) (Parmacek et al., 1989; Quackenbush et al., 1987; Teixeira et al., 1987). Figure 1 shows the structural analogies and the sequence homology between rBAT and 4F2hc proteins. Both proteins lack a membrane leader sequence, have similar hydrophobicity plots (reviewed in Palacín, 1994), and share four regions (10–18 amino acid residues long) one of which is highly conserved (67–80% identity) (Fig. 1). Both proteins also have a domain with significant homology with a protein family of prokaryotes and insect a-amylases and a-glucosidases (Bertran et al., 1992b,c; Wells and Hediger, 1992). Interestingly, the catalytic site of these glucosidases is not totally conserved in rabbit rBAT or human 4F2hc; this is consistent with the fact that expression of rBAT in oocytes does not show a-amylase or maltase activity (Wells and Hediger, 1992).

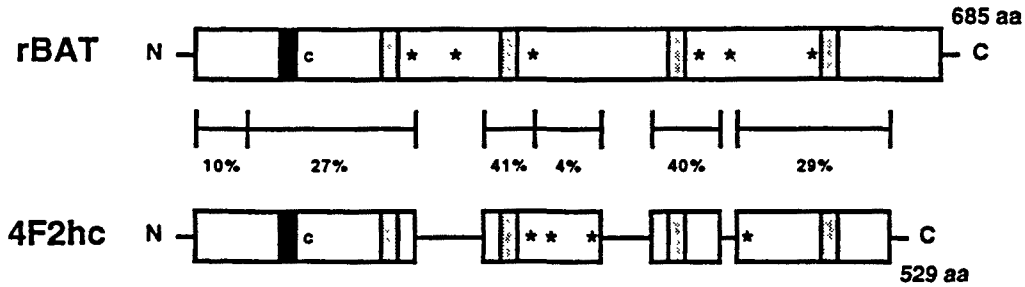

Fig. 1. Schematic representation of the structural analogies and of sequence similarities between the rBAT and 4F2hc human proteins (deduced from the cDNA sequences). Both proteins have only one segment that is clearly a transmembrane domain (black bars in positions 89–110 and 82–104 for rBAT and 4F2, respectively). S. Tate and coworkers (Mosckovitz et al., 1994) propose, for rBAT, the existence of three more transmembrane domains of amphipatic nature. Two of them would also be conserved in 4F2hc. These two proteins show around 30% identity (45% similarity) in their amino acid sequence. This similarity through their sequence is shown in the diagram. Four fragment sequences (10–18 amino acid residues long) are highly conserved (67–80% identity) between both proteins (dotted areas). Human, rabbit and rat cloned rBAT proteins have 8 cysteine residues, while human 4F2hc has two. The first cystine residue in these sequences (positions 114 and 102 for human rBAT and 4F2hc respectively) is conserved (lower c in the diagram). Asterisks show the positions for N-glycosylations. Gaps in this alignment smaller than 10 residues long are not shown. aa stands for amino acid

The cDNA of 4F2hc was cloned using a monoclonal antibody designed against a cell surface antigen from lymphoblastoid cells (Quakenbush et al., 1987; Teixeira et al., 1987). The biological role of this antigen was unknown at that time. Due to rBAT and 4F2hc homology, cRNA from 4F2hc was tested in oocytes for expression of amino acid transport activity. Expression of 4F2hc in oocytes resulted in an amino acid transport activity different from that elicited by rBAT (Bertran et al., 1992b; Wells et al., 1992). Thus, rBAT induces, through the oocyte plasma membrane, transport of cystine, dibasic and neutral amino acids. This is a high-affinity transport with K_m values in the μM range for amino acids such as L-cystine, L-arginine, L-lysine, L-ornithine, L-leucine and L-histidine. The kinetics of L-cystine transport by human rBAT cRNA in oocytes is shown in Fig. 2. Kinetic and cross-inhibition studies (i.e., Dixon plot analysis) provided convincing evidence that rBAT induces a single amino acid transport system in *Xenopus* oocytes (Bertran et al., 1992b), which is not present in stage VI oocytes (Fig. 2, Bertran et al., 1992b,c; McNamara et al., 1991). This transport activity is sodium-independent and it is very similar to the amino acid transport system $b^{0,+}$ defined by Van Winkle's group in mouse blastocysts, as a sodium-independent high-affinity system for dibasic and neutral amino acids (Van Winkle et al., 1988). In contrast to the transport system associated to rBAT, the blastocyst $b^{0,+}$ system does not transport L-cystine (Van Winkle, personal communication). For this reason we named our human and rabbit cDNA clones rBAT, as the acronym for "related to $b^{0,+}$ amino acid transporter). In contrast to rBAT, the cRNA of human 4F2hc seems to increase an amino acid transport activity already present in the

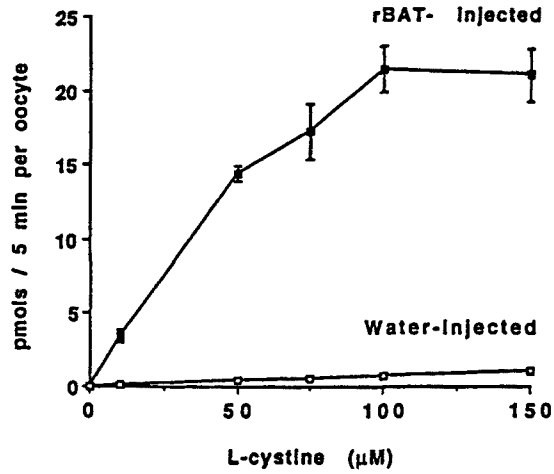

Fig. 2. L-cystine transport induced by human rBAT in *Xenopus* oocytes. Oocytes were injected with 50 nl of water (empty squares) or 50 nl of water containing 5 ng of human rBAT synthetic RNA (black squares). After three days, L-[^{35}S] cystine uptake at the indicated aminoacid concentration was determined during 5 minute incubations. rBAT cRNA induced cystine uptake is saturable at cystine concentrations close to 100μM. In contrast, cystine uptake in control oocytes injected with water does not show saturability in the aminoacid concentration range studied. Values represent the mean ± SEM of uptake of seven oocytes in each group, in a representative experiment

oocytes, which is sodium-independent with high-affinity (μM range) for L-dibasic amino acids, but with high-affinity for L-neutral amino acids only in the presence of sodium; in the absence of sodium the affinity for L-neutral amino acids is dramatically reduced (Bertran et al., 1992c; Wells et al., 1992). This transport activity, which does not transport L-cystine, is very similar to the system y^+L, initially described in human erythrocytes by Devés et al. (1992). Moreover, Ganapathy's group has recently shown that poly(A)+ RNA from a human choriocarcinoma cell line expresses y^+L transport activity in oocytes which is hybrid-depleted by 4F2hc antisense oligonucleotides (Fei et al., 1995).

The rBAT and 4F2hc proteins may be a component of heteromeric amino acid transporters

We have previously discussed the amino acid transport data due to rBAT and 4F2hc expression in oocytes. Are rBAT and 4F2hc real amino acid transporters? If so, their structure should accomplish the translocation of amino acids through a biological membrane. The present paradigm considers that a membrane transporter of substrates of polar nature should be an integral membrane protein with enough hydrophobicity to form a pore through the membrane, generally with 12 transmembrane domains (Wright, 1994).

Biochemical and immunochemical studies have demonstrated that rBAT and 4F2hc are integral membrane N-glycoproteins. The experimental evi-

dences for rBAT are: 1) *translation in vitro.* Addition of microsomes to the reticulocyte translation system increases (<20kDa) the molecular mass of the protein product synthesized from rBAT cRNA (Wells and Hediger, 1992; Markovich et al., 1993). 2) *Expression in oocytes.* The protein product (~90kDa) from rBAT cRNA in oocytes, shown by metabolic labelling with [^{35}S] methionine, is an integral N-glycoprotein. Thus, the product is not solubilized from oocyte membranes by sodium carbonate treatment. The treatment of the oocytes with tunicamycin reduces the size of the protein to ~72kDa in polyacrylamide gels, compatible with the mass of the deduced protein from the cDNA ($Mr < 79 \times 10^3$) (Bertran et al., 1993). 3) *Studies with the native protein.* Western blot analysis using specific anti-rBAT antibodies revealed a protein band of 90–95kDa in membrane preparations from kidney and mucosa from the small intestine (Furriols et al., 1993; Mosckovitz et al., 1993). The size of this band is reduced to ~72kDa after endoglycosidase F treatment of renal brush border membranes (Mosckovitz et al., 1993; Chillarón and Palacín, unpublished results).

The main argument against rBAT and 4F2hc being the only transporter proteins of system $b^{0,+}$-like and y^+L-like, respectively, is the prognosis of their structural organization into the plasma membrane. The hydrophobicity algorithms applied to rBAT and 4F2hc suggested a single transmembrane domain (see legend to Fig. 1). Due to the lack of leader peptide and because the N-glycosylation sites are towards the C-terminus from the localization of the putative transmembrane domain, it was proposed that rBAT and 4F2hc were type II membrane glycoproteins (i.e., cytosolic N-terminus and extracellular C-terminus) (Bertran et al., 1992b; Wells and Hediger, 1992; Quakenbush et al., 1987; Teixeira et al., 1987). In contrast, Tate's group have proposed that rBAT crosses the plasma membrane at least four times, with the first transmembrane domain already mentioned and three additional amphipathic transmembrane domains (Fig. 3). This is based on studies of limited proteolysis and peptide-specific antibody detection of permeabilized cells expressing the rBAT protein (Mosckovitz et al., 1994). These highly interesting results with the rBAT protein await confirmation with different approaches; similar studies on 4F2hc have not been reported. In any case, it does not seem that one, or even four, transmembrane domains are enough to form a polar pore for the passage of amino acids through the plasma membrane. Alternatively rBAT and 4F2hc may be a component of a heteromeric amino acid transporter (Bertran et al., 1992b,c; Wells et al., 1992): rBAT may be an "activator" of silent $b^{0,+}$-like transporters of the oocyte, and 4F2hc may "activate" inactive oocyte y^+L-like transporters. A possible mechanism for this "activation" could be the constitution of holotransporters with subunits present in the *Xenopus* oocytes. This hypothetical mechanism would be similar to the activation of the oocyte a catalytic subunits of the Na^+/K^+ ATPase by the expression of foreign b subunits of the Na^+/K^+ ATPase (Geering et al., 1989). Interestingly, the cell surface antigen 4F2 is a heterodimer (~125kDa) composed of a heavy chain of 85kDa (4F2hc, i.e. the homologous protein to rBAT) and a light chain of 40kDa linked by disulfide bridges (Haynes et al., 1981; Hemler and Strominger, 1982). Unfortunatelly, this light subunit evi-

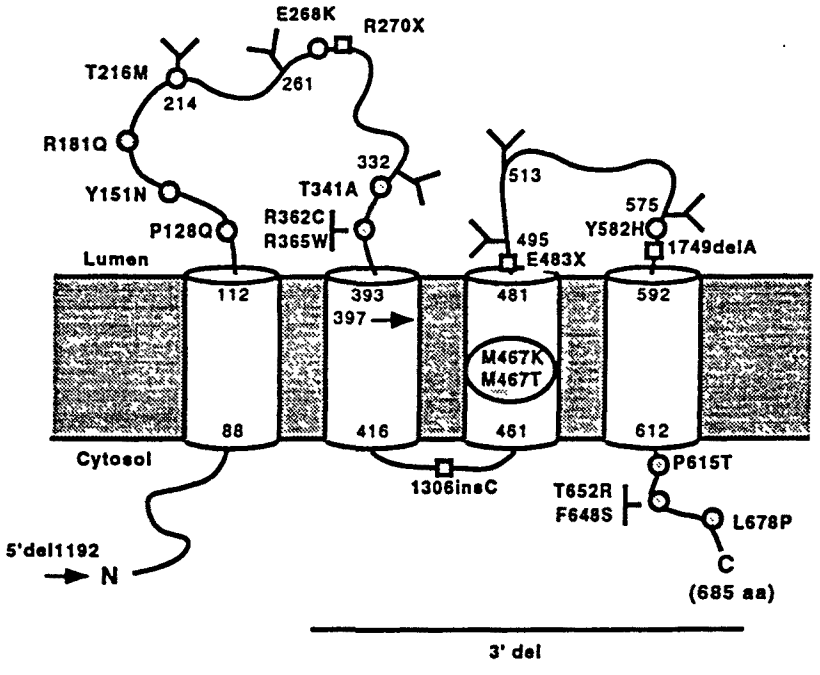

Fig. 3. rBAT protein model with four transmembrane domains as proposed by Tate and coworkers. Three of them would be of amphipatic nature. This model is based on limited proteolysis studies, and the use of different anti-rBAT antibodies in permeable cells (Mosckovitz et al., 1994). Cystinuria-specific rBAT mutations found so far are also shown. Circles represent missense mutations, squares nonsense mutations, and deletions and insertions are represented by "del" or "ins". Arrows give the limits of the 5′ 1192 deletion. Mutations M467T and M467K are found in the third transmembrane domain (in the model proposed by Tate). The other mutations are scattered over the whole protein. Mutation T216M destroys the first potential N-glycosylaton site. Numbers indicate the first and last amino acid residues of the proposed transmembrane domains, and the putative N-glycosylation sites (*Y*). 18 of these mutations have already been published (Calonge et al., 1994; Pras et al., 1995; Gasparini et al., 1995; Miyamoto et al., 1995; Horsford et al., 1995). Mutations Y151N, T216M, R362C, E483X and a 3′ deletion affecting at least from the 5th to the 10th exon (shown by a horizontal line in the scheme) have recently been found by our cystinuria study consortium (manuscript in preparation). Horsford et al. (1995) have reported two additional mutations in one French Canadian cystinuria patient, a genomic rearrangement and a 5′ splice site mutation (1500 + 1 G – T) (not shown in the scheme)

denced by ^{125}Iodine labelling and immunoprecipitation has not been microsequenced or cloned. In a similar way, renal rBAT is immunodetected in Western blot studies in non-reducing conditions as complexes of ~240kDa and ~125kDa; in two-dimensional gels (first with non-reducing conditions, followed by reducing conditions) the 240kDa and the 125kDa bands contribute to the ~90kDa seen in reducing conditions (Chillarón and Palacín, unpublished results). Very recently, Tate's group has reported the presence of these complexes in brush border preparations from kidney and intestine (Wang and Tate, 1995). Interestingly, in membranes obtained in the presence of N-ethyl maleimide from oocytes expressing rBAT, complexes similar in

size to the ones observed in kidney have been reported (Wang and Tate, 1995). All this suggests rBAT's similarity to the 4F2 antigen, hence rBAT may form a heterodimeric structure (125kDa) of a "heavy chain" (90kDa) linked by disulfide bridges to a putative "light chain" of 40–50kDa. If the hypothesis of the heterodimeric holotransporters for rBAT and 4F2 is shown to be valid, the amino acid transport systems $b^{0,+}$-like and y^+L-like will be the first examples of heteromeric transporters for organic substrates in mammals. Knowledge of the structure-function relationship of rBAT and 4F2hc will need the isolation and cloning of the light chain of 4F2 and the putative light chain of rBAT.

The role of rBAT in the active reabsorption of cystine and dibasic amino acids in kidney

The rBAT mRNA is expressed in the kidney and the mucosa of the small intestine (Bertran et al., 1992 a,c; Lee et al., 1993; Wells and Hediger, 1992; Yan et al., 1992). In agreement with this, hybrid-depletion with rBAT antisense oligonucleotides blocks expression of system $b^{0,+}$-like transport by renal and intestinal poly(A)$^+$ RNA in oocytes (Wells and Hediger, 1992; Magagnin et al., 1992; Bertran et al., 1993). Northern blot analysis of renal and intestinal RNA revealed two rBAT transcripts; ~2.3kb and ~4kb in length. A cDNA corresponding to the long rBAT transcript was identified by expression cloning in oocytes, and represents an alternative polyadenylation of the same gene (Markovich et al., 1993). *In situ* hybridization and immunolocalization studies have demonstrated that rBAT localizes to the microvilli of the small intestinal mucosa and the epithelial cells of the proximal straight tubules (S3 segment) of the nephron (Kanai et al., 1992; Furriols et al., 1993; Pickel et al., 1993). In addition to kidney and intestine, brain tissues show a transcript of ~5kb that hybridizes with rBAT cDNA probes (Bertran et al., 1992b, 1993; Yan et al., 1992). RNA protection assay studies and Western blot analysis with anti-rBAT peptide antibodies suggested that this long transcript corresponds to the expression of a gene homologous to rBAT (Pickel et al., 1993; Yan et al., 1992).

The presence of the protein rBAT in the microvilli of the epithelium of kidney and intestine suggested a role of rBAT in the renal and intestinal reabsorption of amino acids. The question arises as to how a sodium-independent transporter like the $b^{0,+}$-like system associated with rBAT participates in a reabsorption process? In other words, what is the concentrative mechanism for amino acid reabsorption of system $b^{0,+}$-like activity? The answer to this question came from two different lines of research: through the study of the electrical activity of rBAT transporter expression in oocytes, and through the association of rBAT gene mutations with cystinuria, a common inherited amino aciduria (see below). The group of Andreas Busch and Florian Lang at the University of Tübingen studied the electric activity of the system $b^{0,+}$-like activity expressed by rBAT in oocytes (Busch et al., 1994). The initial results were unexpected and are shown in Fig. 4. In oocytes expressing rBAT (not in control oocytes injected with water) the presence of

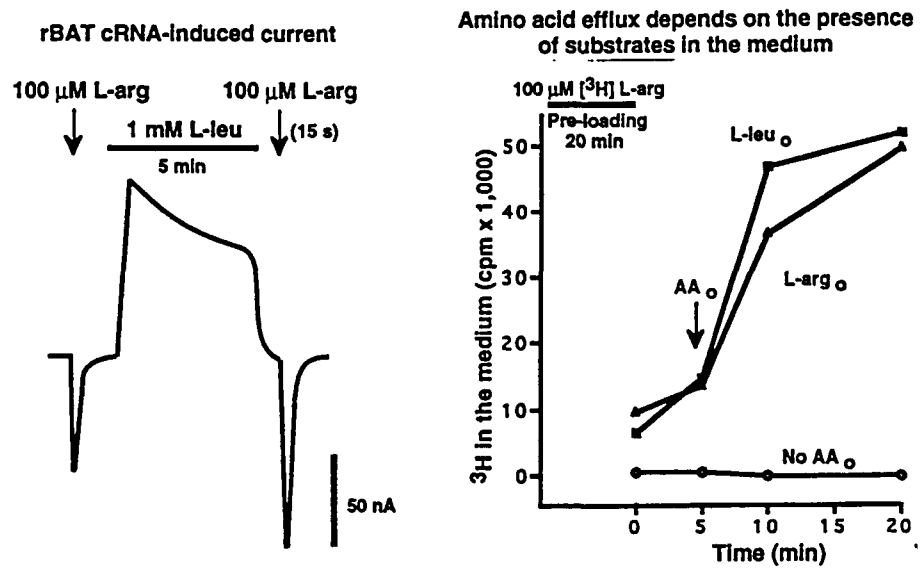

Fig. 4. The transport activity induced by rBAT cRNA in *Xenopus* oocytes is that of an obligatory amino acid exchanger, with substrate specificity similar to the $b^{0,+}$ system. rBAT cRNA expression (1 ng per oocyte) induces an inflow current of positive charge when there is arginine in the medium (left panel). Surprisingly, in the presence of leucine (zwitterionic amino acid that is neutral at pH of 7), the induced current is an efflux of positive charges. These representative results were obtained from an oocyte with its transmembrane potential set at −40 mV, three days after rabbit rBAT RNA injection (Busch et al., 1994). To confirm that the basic amino acid movement, an exchange mechanism through the rBAT/$b^{0,+}$-like transporter, was responsible for the induced current, amino acid efflux experiments were performed in the presence of rBAT substrates in the oocyte incubating media (right panel). Oocytes were injected with rabbit rBAT RNA (1 ng per oocyte). After three days, the oocytes were incubated in the presence of L-[^{3}H] arginine (100 μM) for 20 min. After washing out the radioactive medium of the oocytes, tritium efflux to the medium was measured (in these conditions more than the 95% of tritium corresponds to L-[3H] arginine) (Chillarón et al., 1995). This arginine efflux is dependent on the presence in the medium of substrates of the transporting system associated to rBAT (either leucine or arginine in the figure). In the absence of substrates in the medium (no amino acids), arginine efflux is minimal, and the same as that found in oocytes injected with water, both in the presence or absence of substrates in the medium (Chillarón et al., 1995)

L-arginine in the medium produces an inward positive current, most probably due to the positive charge of arginine at neutral pH. Surprisingly, exposure of rBAT-expressing oocytes to L-leucine produce an outward positive current through the plasma membrane of the oocyte. The participation of inorganic ions (e.g., K^+, Cl^-) in these currents was eliminated. These results prompted the hypothesis that the $b^{0,+}$-like/rBAT transporter exchanges amino acids through the plasma membrane: the outward positive current produced by neutral amino acids (e.g., L-leucine) would be due to the concomitant exit of dibasic amino acids from the oocyte. To examine this hypothesis, the dependence on external amino acids of the efflux of amino acids from oocytes expressing rBAT was tested. Figure 4 shows that efflux of L-[^{3}H] arginine is

totally dependent on the presence of amino acids in the medium. These results have been confirmed by others (Coady et al., 1994; Ahmed et al., 1995). In fact, Coady's group has isolated a renal rBAT cDNA by expression of the electric activity of system $b^{0,+}$-like/rBAT in oocytes. Additional data confirmed that the rBAT induced activity is an obligatory exchanger. Thus, the amino acids that elicited efflux from the oocyte are the substrates of system $b^{0,+}$-like /rBAT activity, and the apparent K_m for stimulating efflux is the same as the K_m for influx studies (Chillarón et al. 1995). Very recently, we have demonstrated that system $b^{0,+}$ -like activity is also present in a "renal proximal tubular" cell line (i.e., the opossum kidney cell line OK): the transport of cystine in the apical pole is shared with dibasic and neutral amino acids, shows complete dependence of substrate efflux on external amino acids, and this transport activity is due to the expression of the rBAT gene (Mora et al., 1995). At present the stoichiometry and the mechanism of exchange associated with rBAT remain to be established.

An obligatory exchanger could be considered as a tertiary active transporter, which exchanges gradients of substrates through the membrane. In other words, system $b^{o,+}$ -like activity should be capable of accumulating amino acids by dissipating the pre-existing gradient of a given amino acid. In fact, oocytes expressing rBAT are able to accumulate, in a sodium-independent manner, the amino acids L-cystine (in conditions that prevent intracellular reduction to cysteine), L-arginine and L-leucine 30 to 60-fold versus the external medium; these levels of accumulation are 5 to 30 times higher (depending on the substrate) than the accumulation that occurs in control oocytes (Chillarón et al., 1995). The membrane potential (−50 to −65 mV in oocytes expressing rBAT) is not able to hold these gradients; it is able to maintain a gradient of 7 to 14 fold for an amino acid with a positive charge, but this does not explain the accumulation of L-leucine and L-cystine. On the other hand, oocytes contain a very high intracellular concentration of amino acids, which has been estimated to be approximatelly 2,500 μM neutral amino acids and 750 μM dibasic amino acids (Taylor and Smith, 1987). This intracellular concentration of amino acids is enough to couple the accumulation achieved by the rBAT substrates in oocytes.

Taking into account the cellular localization of the rBAT protein and its mechanism of tertiary active transport we propose a model for the physiological role of thes system $b^{0,+}$-like transporter in the renal reabsorption of cystine and dibasic amino acids (Fig. 5). In this model the function of the transporter is directed towards apical reabsorption of cystine and dibasic amino acids, which dissipates the intracellular gradient of neutral amino acids. The negative membrane potential and the intracellular reduction of cystine to cysteine should favour this sense of the exchange. Neutral amino acids into the tubular lumen should then be reabsorbed via active transporters (e.g., the sodium-dependent system Neutral Brush Border) located in the apical plasma membrane of tubular epithelial cells. Is this model valid? The fact that mutations in the rBAT gene cause cystinuria, aminoaciduria of cystine and dibasic amino acids, but not neutral amino acids, argues in favour of this hypothesis (see below).

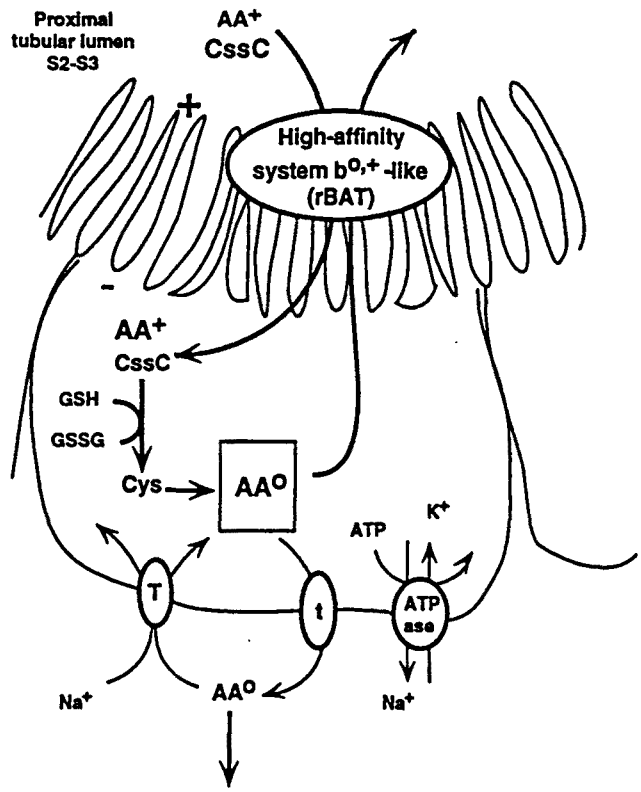

Fig. 5. Model proposing the role of the transport system rBAT/$b^{0,+}$-like in the reabsorption of cystine and dibasic amino acids in the kidney. The rBAT protein would be part of the renal reabsorption system of cystine and dibasic amino acids of high-affinity occuring in the nephron S3 segment. Thus, rBAT would be responsible for 15–20% of the tubular cystine reabsorption in the kidney, which would be of high-affinity would occur at the most distal segment of the proximal tubule (Silbernagl, 1988). Dibasic amino acids and cystine concentration through the apical membrane of the cells would occur by an active tertiary transport mechanism, linked to a high concentration of neutral amino acids inside the cell. Moreover, the entrance of dibasic amino acids and cystine from the nephron tubule lumen would be favoured by the membrane potential and by the reduction of cystine to cysteine, respectively. High neutral amino acid concentrations in the interior of the epithelial cells would be due to the concentrating neutral amino acid transport activities in the apical pole (cotransport system with Na^+, Neutral Brush Border) and the basolateral pole (Systems ASC, etc.; T transporters shown in the diagram), linked to the Na^+ electrochemical gradient, established by the basolateral activity of the Na^+/K^+ ATPase. Other basolateral transporters, either for dibasic (not shown) or neutral (L system type: t transporters in the diagram), would ensure amino acids efflux to the organism. This model is based on the active tertiary transport mechanism (amino acid exchanger) seen in *Xenopus* oocytes and OK cells, and on the rBAT localization in the microvilli of the epithelial cells of the S3 segment of the nephron

Identification of rBAT as a cystinuria gene

Classic cystinuria is an autosomic recessive disease with a prevalence of 1 in 7,000 newborns. It is characterized by urinary hyperexcretion of cystine and dibasic amino acids (Levy, 1973; McKusick, 1990; Segal and Thier, 1995). Due

to the poor solubility of cystine, it precipitates to form kidney calculi that produce obstruction, infection and ultimately renal insufficiency (Segal and Thier, 1995). Three types of classic cystinuria have been described (Rosenberg et al., 1966a): type I heterozygotes present normal amino aciduria, whereas type II and III heterozygotes present high and moderate hyperaminoaciduria of cystine, lysine, and to a lesser extent, of arginine and ornithine. As a consequence of the intestinal amino acid transport defect, type I and II homozygotes do not show increases in the plasma level of cystine after an oral administration of the amino acid. In contrast, type III homozygotes show a nearly normal increase in the plasma level of cystine after the oral dose. Genetic studies suggested that the three types of cystinuria were due to allelism of a single gene (Rosenberg et al., 1966b).

Dent and Rose (1951) postulated that cystinuria may result from defective function of a common uptake system for cystine and dibasic amino acids. Milne et al. (1961) demonstrated a reduced intestinal absorption of dibasic amino acids in patients with cystinuria. Finally, in vitro transport studies demonstrated a defective accumulation of cystine and dibasic amino acids in biopsies of patients with cystinuria (Thier et al., 1964; Coicadan et al., 1980). Unfortunately, the present knowledge of cystine reabsorption in kidney and intestine is incomplete and unclear. Work with brush border membrane perparations from rat kidneys suggested that L-cystine reabsorption is mainly sodium-independent (Foreman et al., 1980; McNamara et al., 1981, 1982). In the absence of any energy-coupled mechanism, cystine and dibasic amino acids would be accumulated through the apical membrane of kidney epithelial cells because of the intracellular reduction of cystine to cysteine and the negative membrane potential, respectively; basolateral transport systems would mediate the efflux of these amino acids (Silbernagl, 1988). Segal's group have provided evidence that renal brush border membrane vesicles show two cystine transport systems; one with high-affinity (K_m in the μM range), shared with dibasic amino acids, which shows heteroexchange diffusion, and the other of low-affinity and not shared with dibasic amino acids (Segàl et al., 1977; McNamara et al., 1981). In addition, several authors have found inhibition by neutral amino acids of cystine uptake, measured at low concentration (μM range) in renal brush border preparations or perfused tubules, suggesting that the high-affinity system is also shared with neutral amino acids (Foreman et al., 1980; Schafer and Watkins, 1984; Furlong and Posen, 1990). Very recently, we have demonstrated that cystine is transported in the apical pole of the "renal proximal tubular" cell line OK by a sodium-independent, high-affinity transport system, shared with dibasic and neutral amino acids with characteristics of system $b^{0,+}$-like (Mora et al., 1995). In contrast to renal preparations, cystine transport in brush border from mucosa of the small intestine shows a single kinetic transport system of high-affinity, shared with dibasic amino acids (Ozegoic et al., 1982). Therefore, this high-affinity system, present in kidney and intestine may be the system that is defective in cystinuria (Thier et al., 1964; Coicadan et al., 1980). Microperfusion studies showed that this cystine high-affinity transport system is present in the proximal straight tubule (S3 segment), whereas the low-affinity system is

present in the proximal convoluted tubule (S1–S2 segments) (Schafer and Watkins, 1984).

The specific expression of rBAT in the microvilli of the S3 segment of the nephron and in the mucosa of the small intestine, and the transport induced by rBAT in oocytes, suggested that the system $b^{0,+}$-like transporter (associated with rBAT) is an active (i.e., tertiary active transport) reabsorption system of high affinity for cystine and dibasic amino acids. This information supports the hypothesis of rBAT as a good candidate gene for cystinuria.

This hypothesis was tested by mutational analysis of the rBAT gene in patients with cystinuria. Initially we did not know the genomic structure of the rBAT gene. To circumvent the use of renal or intestinal biopsies we took advantage of illegitimate transcription: any gene is expressed in any cell type but at very low copy number (Chelly et al., 1989). Lymphoblastoid cell lines from patients with cystinuria were developed. Following the amplification of the rBAT mRNA of these cells by RT-PCR, single stranded-chain polymorphism analysis and DNA sequence, six missense mutations in the rBAT gene that co-segregate with the cystinuria phenotype were identified (Calonge et al., 1994). Functional analysis in oocytes, of the most common mutation found (M467T; substitution of methionine residue 467 for threonine) showed a reduced transport activity of system $b^{0,+}$-like. These results provide strong evidence that mutations in the rBAT gene cause cystinuria (Calonge et al., 1994). Later, we obtained complete information on the genomic structure of rBAT (i.e., exon-intron boundaries) that permits direct mutational analysis of the gene from DNA isolated from blood of patients (manuscript in preparation). Mutational analysis with this material is providing a growing number of new cystinuria-specific mutations in the rBAT gene (at present we have found seven additional mutations, including insertions, deletions, stop codon and missense; four of these mutations are described in Gasparini et al., 1995). These data have been confirmed by others: Pras and coworkers (1995) have reported four new cystinuria-specfic mutations (one stop codon, two deletions and one missense) in populations in the Middle East and Eastern Europe, mainly of Jewish origin, and Miyamoto and coworkers (1995) have reported two new missense mutations in Japanese cystinuric patients that showed reduced amino acid transport expression in oocytes. At present the most frequent cystinuria-specific mutations found in the Italian, Spanish and Jewish populations are M467T and R270X (new stop codon instead of arginine at residue 270; this eliminates 2/3 of the protein towards the C-terminus). These mutations have been found in several homozygote patients and in compound heterozygotes with other mutations. The mutations known at present are described in Fig. 6; their localization in the protein is shown in Fig. 3. In summary, strong evidence has been offered for a role of rBAT in cystinuria.

Very recently, we obtained evidence that the system $b^{0,+}$-like activity, which mediates the apical transport of cystine, in the apical pole of the "renal proximal tubular" cell line OK is due to the expression of rBAT; expression of antisense rBAT sequences specifically reduces this amino acid transport activity in OK cells (Mora et al., 1995). This is the first direct demonstration

Cystinuria-specific mutations in the rBAT gene

Mutations	Exon	Independent chromosomes	
		number	origin
Missense			
P128Q	1	4	2 Persian Jews, 2 Yemenite Jews
Y151N	2	1	Italian
R181Q	2	1	Italian
T216M	3	2	Italian
E268K	4	1	Japanese
T341A	6	1	Japanese
R362C	6	1	Italian
R365W	6	1	Italian
M467K	8	1	Italian
M467T	8	13	6 Italians, 6 Spanish 1 Canadian
Y582H	9	1	Italian
P615T	10	1	Italian
F648S	10	1	Italian
T652R	10	1	Italian
L678P	10	1	Italian
Stop codon			
R270X	4	11	2 Druze, 1 Italian 8 Ashkenazi Jews
E483X	8	2	2 Italians, 1 French Canadian
Splice mutation			
5' site			
1500 + 1 G-T	8–9	1	1 French Canadian
Deletions and insertions			
1749delA	10	1	Italian
5'del1192?	1 al 6	1	EQt European
1306insC	7	1	EQt European
3'del?	5 al 10	1	Italian

Fig. 6. Cystinuria-specific rBAT gene mutations. The exon in which each mutation occurs is stated, and also the number and origin of independent chromosomes in which they were found. To date 23 mutations have been discovered that segregate with the classic cystinuria phenotype: 15 missense mutations in well conserved amino acid residues of the human, rabbit and rat rBAT gene, 2 nonsense, 1 splice site, 4 deletions or insertions and 1 genomic rearrangement (Horsford et al., 1995; not shown in the list). For two of the deletions the limits are still not completely defined, so they are marked with a question mark. Five mutations have been found in more than one independent chromosome: M467T, R270X, P128Q, E483X and T216M. These mutations have been described by Calonge et al. (1994) and Gasparini et al. (1) for the ones with Spanish or Italian origin, by Pras et al. (1995) for the ones with Jewish, Druze and East European origin, by Miyamoto et al. (1995) for the ones with Japanese origin, and by Horsford et al. (1995) for the ones of Canadian origin. Report of mutations Y151N, T216M, R362C, E483X, and the 3' deletion of Italian origin is at present in preparation

that rBAT is associated with system $b^{0,+}$-like activity in mammalian cells. This and the cystinuria-specific mutations found in the rBAT gene allows us to propose that system $b^{0,+}$-like activity (associated with rBAT) participates in the renal and intestinal reabsorption of cystine and dibasic amino acids of high-affinity, most probably with a tertiary active transport mechanism as indicated in the model shown on Fig. 5. Due to the localization of rBAT in the S3 segment of the nephron, where only 15–20% of cystine reabsorption occurs (Silbernagl, 1988), system $b^{0,+}$-like could be envisaged as a low-capacity high-affinity system of marked physiological importance as revealed by its alteration in cystinuria.

Genetic heterogeneity in cystinuria

Having demonstrated that rBAT is a cystinuria gene, the question now arises as to whether it is the only one, or whether classic cystinuria is a genetically heterogeneous disease. Is rBAT responsible for all three types of cystinuria? Clinical and physiological evidence suggested heterogeneity in cystinuria: i) most of renal reabsorption of cystine (80–85%) occurs in the S1–S2 segments of the nephron (i.e., in different tubular region in which rBAT is expressed). ii) The oral cystine test may indicate that in type III cystinuria the intestinal defect is not very conspicuous. iii) Severity of cystinuria in newborn type I, but not type III, heterozygous probands ameliorates within the first year of life, suggesting that ontogeny of renal L-cystine reabsorption amplifies phenotypic expression of cystinuria alleles (Scriver et al., 1985). iv) Goodyer and coworkers (1993) observed extremely high urinary cystine levels in type I/I homozygous children whereas cystine excretion by type I/III infants was lower, suggesting complementation between two different genetic loci for type I and type III cystinuria. Thus, other cystine reabsorption system(s) not present (or not very conspicuous) in the small intestine may also be encoded by cystinuria gene(s).

We then obtained an indication of genetic heterogeneity for cystinuria: cystinuria-specific mutations in the rBAT gene were present only in patients with type I cystinuria (Gasparini et al., 1995). Thus, when the access to the rBAT gene from blood samples covered 70% of the coding region we found rBAT mutations in 50%, 8 out of 16 type I chromosomes, in 33%, 3 chromosomes out of 9 type I chromosomes inherited as compound heterozygotes with type III chromosomes; no mutations were found in a total of 37 type II or type III chromosomes, including those inherited together with type I. At present, having analysed the whole coding region of the rBAT gene, cystinuria-specific mutations have been found in ~75% of type I chromosomes. Similarly, Goodyer's group have found cystinuria-specific mutations in rBAT only in type I cystinuria chromosomes from type I/I or type I/III cystinuria patients (Horsford et al., 1995).

The easiest way to test for genetic heterogeneity in cystinuria, and confirm that rBAT is responsible for only type I cystinuria, is to perform genetic linkage studies with markers of the rBAT genomic locus. Initially the rBAT gene was localized to the short arm of chromosome 2 by using somatic hybrid

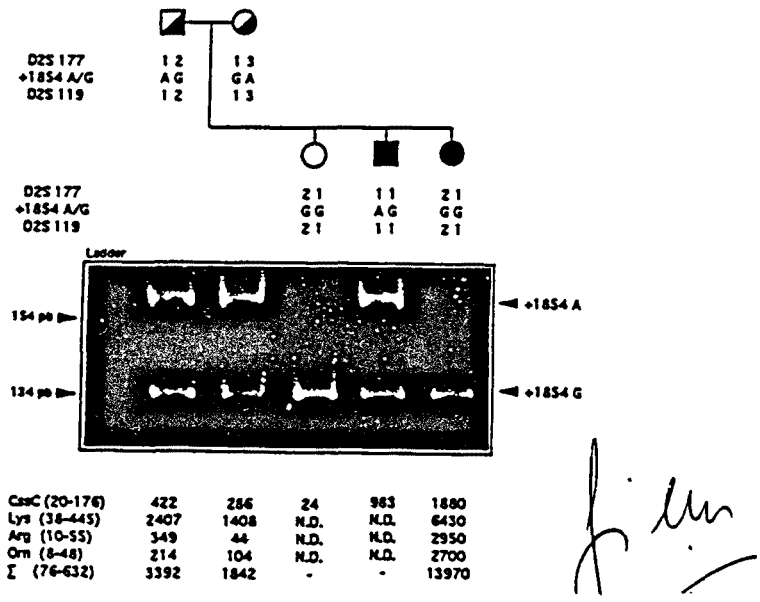

Fig. 7. Family with cystinuria type III/III showing no genetic disequilibrium with the rBAT locus. This family is taken from a study in which genetic heterogeneity linked to cystinuria type III is shown (Calonge et al., 1995b). Filled and half filled symbols represent cystinuric patients and carriers respectively. Under the symbols are shown the haplotypes made by the genotypes of microsatellite D2S177, and rBAT polymorphism and microsatellite D2S119. Under the gel picture are shown the urinary excretion values (in m mol g^{-1} creatinine), for cystine (CssC), arginine (Arg), lysine (Lys), ornithine (Orn), and the sum of all of them (S). Normal urinary excretion values for these amino acids are shown in brackets

cells (Lee et al., 1993; Calonge et al., 1994). Independently, it was found that microsatellites from the short arm of chromosome 2 (e.g., microsatellite D2S119) were linked to cystinuria phenotype in families from the Middle East and of Jewish origin (Pras et al., 1994). To test if this linkage locus was the rBAT gene, a mega YAC (yeast artificial chromosome) clone containing the human rBAT gene (insert >1Mb long) was isolated from the human mega YAC library from CEPH (Dr. Le Paslier, France). Alu PCR-amplified human sequences from this clone were used to localize the rBAT gene by fluorescence *in situ* hybridization to the G-band 2p16.3 in chromosome 2; similarly the positive linkage chromosome 2 markers for cystinuria (i.e., D2S119 and D2S177; Pras et al., 1994) also localized to the same G-band as the rBAT gene (Calonge et al., 1995a). Others have localized rBAT in the nearest chromosome 2 G-band 2p21 (Yan et al., 1994). This confirmed the identity between the rBAT gene and the cystinuria locus defined by linkage studies. Next, these chromosome 2 markers (i.e, D2S177 and D2S119) as well as intragenic rBAT markers (i.e., polymorphisms and cystinuria-specific mutations) were used in genetic linkage studies with families transmitting cystinuria types I and III separately or together. Cystinuria types were classified depending on the urinary aminoaciduria values of the obligatory cystinuria carriers. Linkage was only positive with type I/I families, whereas it was negative (i.e., total lod

score value <-20) for type I/III or type III/III cystinuria families (Calonge et al., 1995b). Figure 7 shows the lack of co-segregation between rBAT locus markers (i.e., microsatellites D2S119 and D2S177, and a rBAT polymorphism) and cystinuria in a type III/III family. Unpublished results from our group also demonstrate lack of co-segregation, in two families, between the rBAT locus and cystinuria type II, the least frequent type of cystinuria (manuscript in preparation). Taking together the data demonstrates that, in contrast to the widely held opinion (McKusick, 1990), cystinuria is an inherited disease with genetic heterogeneity; at present it seems that rBAT is only responsible for type I cystinuria, but not types II or III. Studies with more cystinuria families and a better phenotypic classification of the cystinuria types will be needed to establish this point completely. In addition, the fact that compound heterozygotes with type I/III cystinuria have mutations in rBAT for type I but not for type III cystinuria strongly suggests phenotypic interaction between the rBAT gene and the putative type III cystinuria gene.

Candidate genes for cystinuria

Further perspectives

The scheme of Fig. 8 summarizes the present knowledge of the molecular biology of cystinuria. Type I cystinuria, the most frequent in our populations (i.e., >60% of the cases) is due to mutations in the rBAT gene. This gene codes for a protein that most probably participates as a modulator or as the catalytic part of a heterodimeric $b^{0,+}$-like transporter. This activity is responsible for the high-affinity cystine and dibasic reabsorption in the S3 segment of the nephron and in the small intestine, with a tertiary active transport mechanism coupled to the exchange of neutral amino acids. Recently it has been demonstrated that this transport activity is due to rBAT expression in OK-cells, a "renal proxinal tubular" cell line (Mora et al., 1995); additional studies are needed to characterize system $b^{0,+}$-like transport activity in renal and intestinal tissues. The putative "light subunit" of rBAT could also be envisaged as a type I cystinuria gene. In contrast the gene(s) causing cystinuria types II and III are unknown. The type III cystinuria gene is likely to have little or no expression in the small intestine. The transport system(s) responsible for the high-capacity low-affinity reabsorption of cystine in the S1–S2 segments of the nephron are obvious candidates to be defective in cystinuria type III.

As far as we know four main lines of cystinuria research are in progress. 1) Design of animal models to study the pathophysiology and for the development of new therapies for this disease. The present therapy reduces cystine excretion and increases calculus solubililty or disagregation, which is accompanied by many secondary effects. This will be further clarified by studies in mice that lack rBAT activity, when the gene is "knocked out". Such efforts are in progress. 2) Identification of new cystinuria genes, by genetic approaches (i.e., exclusion map) or biochemical approaches (i.e., cloning of new renal

The Molecular basis of cystinuria

Phenotype	Gene	Transport system	Affected tissue
I	rBAT	Amino acid exchanger ($b^{0,+}$-like)	Kidney (S3 segment) Small intestine
II	?	?	Kidney & Small intestine
III	?	Low-affinity reabsorption system?	Kidney (S1–S2 segment)?

Fig. 8. Summary of the present knowledge of the molecular basis of cystinuria. rBAT mutations have been found only in cystinuria type I. rBAT protein is by itelf, or together with other proteins, the system for the amino acid transport $b^{0,+}$-like, responsible for the reabsorption of cystine and dibasic amino acids in the S3 segment of the nephron and the mucosa of the small intestine. The genes and the transport activity responsible for cystinuria type II and III are not yet known. As a working hypothesis we propose a reabsoption system for cystine and dibasic amino acids with high-capacity and low-affinity, located in the epithelial cells of the proximal tubule in the S2 and S3 segments, and not present (or not very conspicuous) in the small intestine, which would be responsible for type III cystinuria. The fact that rBAT can have a heterodimeric structure with a light subunit, not yet cloned, leads as to believe that mutations in this subunit may be responsible for cystinuria type II, or alternatively type I

transporters for cystine). 3) Both rBAT and 4F2hc may need accompanying subunits for full transport activity. The purification and cloning of these subunits is essential to the understanding of the structure-function relationship and the mechanisms of action of these amino acid transporters. 4) Besides the ontogenic regulation of rBAT gene expression (Furriols et al., 1993) nothing is known about the regulation of system $b^{0,+}$-like activity. Understanding the machanisms involved in the regulation of this transport activity could help in the development of new therapies for those cystinuria patients with moderate defects in the $b^{0,+}$-like transport activity. The study of rBAT and 4F2hc offers very interesting and new problems in the field of mammalian amino acid transport across plasma membranes.

Acknowledgements

We thank Dr. Carol MacLeod for the interesting meeting that she organized last August within the 4th International Congress on Amino Acids in Vienna, which was the origin of this review. The editorial help of Robin Rycroft is acknowledged. We also thank all the other groups collaborating in the study of rBAT and the molecular basis of cystinuria: the physiology group of Drs. Heini Murer and Jürg Biber (University of Zurich), the electrophisiology group of Drs. Florian Lang and Andreas Busch (University of Tübingen), and the clinical groups of Drs. Alberto Ponzone (Università degli Studi di Torino, Italy), Pedro Barceló and Ferran Rousaud (IUNA-Fundació Puigvert, Barcelona, Spain), Michelle Galucci, Franco Di Silverio and Gianfranco Rizzoni (Ospedale Cristo Re and Ospedale Bambino Gesú, Roma, Italy). Our most grateful thanks to the cystinuria

patients that have collaborated in our studies. J. C., MJ. C. and R. E. are recipients of pre-doctoral fellowships from CIRIT (Generalitat de Catalunya) and C. M. and J. P. are recpients of pre-doctoral fellowships from Ministerio de Educación y Ciencia and Fundación Pi i Sunyer (Spain), respectively. The work from our labs reviewed here has been supported in part by Direccion General de la Investigación Científica y Técnica, Spain (Grants PB90/0435 and PB93/0738), by the Institut Català de Recerca, by the Fundació Pi i Sunyer and Direcció General de Recerca (GR94-1040) from Catalunya, Spain, and by Ministero Italiano della Sanità and Telethon 94 (grant E083) from Italy.

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Received January 30, 1996

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FAC. BIOLOGIA
BIBLIOTECA

Treball de revisió 2

The molecular basis of renal cystine reabsorption and cystinuria

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Nova Acta Leopoldina (1998) **306**, 55-56.

El doctorand va participar en la correcció i revisió de l'article.

Nova Acta Leopoldina NF *78*, Nr. 306, 55–56 (1998)

The Molecular Basis of Renal Cystine Reabsorption and Cystinuria

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Abstract

The protein rBAT, expressed in the apical plasma membranes of the renal straight proximal tubules and the small intestinal epithelium, induces amino acid exchanger activity with characteristics of system $b^{o,+}$ (i. e., high-affinity sodium-dependent transport for cystine, dibasic and neutral amino acids) in oocytes (CHILLARÓN et al. 1996, MORA et al. 1996). This pointed *rBAT* as a candidate gene for cystinuria, an inherited hyperaminoaciduria of cystine and dibasic amino acids. *rBAT* (chromosome 2p16.3-p21) has been established as a gene for cystinuria type I (a type of cystinuria where the heterocygotes are silent) (CALONGE et al. 1994, 1995). A new locus for cystinuria type III or II (types with non silent heterocygote) has been identified in 19q13.1 (BISCEGLIA et al. 1997). rBAT/system $b^{o,+}$-like and 4F2hc (an rBAT homologous protein)/amino acid transport system y^+ L-like are heterodimeric, composed of the identified »heavy chain« (rBAT or 4F2hc) and unidentified »light chains«, which are disulfide bound. This heterodimers might be the functional units of these transporters:

- the cystinuria-specific rBAT mutant, Met467Thr revealed a trafficking defect to the oocyte plasma membrane, and that < 10% of the rBAT protein in the oocyte plasma membrane is enough to saturate the induced transport activity (CHILLARÓN et al. 1997).
- Dissociation between oocyte surface protein and induced amino acid transport has been obtained also with 4F2hc; in addition, there is co-expression of system y^+ L-like activity upon injection of 4F2hc and rat-lung 1.4–2.4 kb poly(A)$^+$ RNA (ESTÉVEZ et al. submitted).
- System y^+ L-like/4F2hc transport activity is sensitive to SH-group reagents. Interestingly, a cys-less 4F2hc mutant induces y^+ L-like transport activity in oocytes that is also sensitive to these reagents. All this suggests that a limiting factor in oocytes (the »light subunit«?) is necessary for the expression of these transport activities. Purification of the rBAT complex, co-expression cloning of the 4F2hc »light subunit« and 19q cystinuria locus positional cloning are currently under progress to identify the putative »light subunits« of rBAT and 4F2hc and new cystinuria genes.

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Treball de revisió 3

Molecular Biology of mammalian plasma membrane amino acid transporters.

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Physiological Reviews (1998) **78**, 969-1054.

El doctorand va escriure el capítol IB, va participar de forma activa en la búsqueda d'informació i també en la correcció i revisió de l'article.