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

Review Article

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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 ~40kDa 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: rBAT – SLC3A1 gene – Cystinuria – System $b^{0,+}$-like

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, β-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 α-amylases and α-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 α-amylase or maltase activity (Wells and Hediger, 1992).

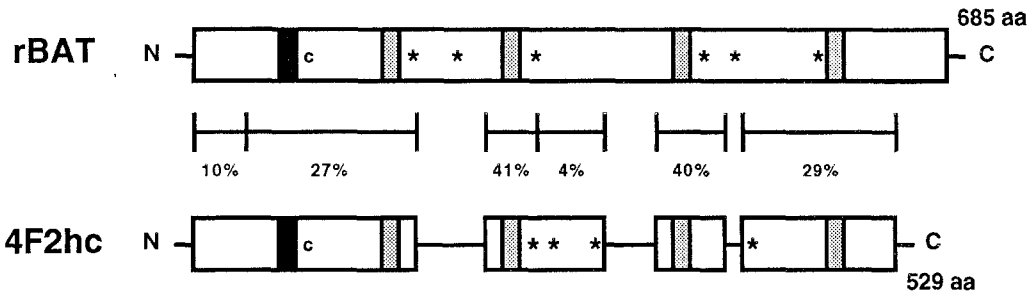

Fig. 1. Schematic representation of the structural analogies and of sequence similarities between the rBAT and 4F2hc human proteins (deduced from the cDNA sequences). Both proteins have only one segment that is clearly a transmembrane domain (black bars in positions 89–110 and 82–104 for rBAT and 4F2, 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

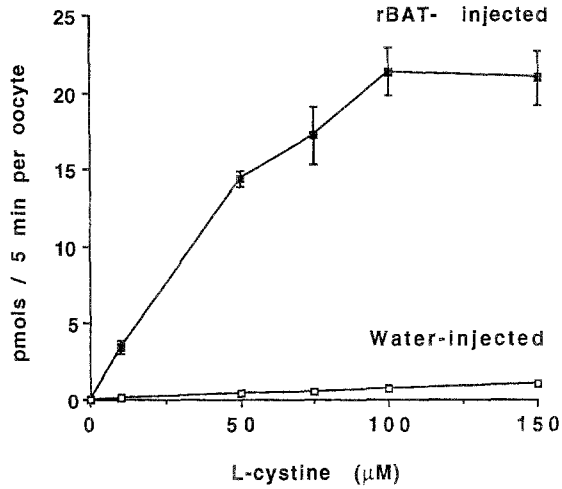

Fig. 2. L-cystine transport induced by human rBAT in *Xenopus* oocytes. Oocytes were injected with 50nl of water (empty squares) or 50nl of water containing 5ng of human rBAT synthetic RNA (black squares). After three days, L-[^{35}S] cystine uptake at the indicated amino acid concentration was determined 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 amino acid 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) *in vitro translation.* Addition of microsomes to the reticulocyte translation system increases (<20 kDa) 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 (~90 kDa) 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 ~72 kDa 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 90–95 kDa 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 ~72 kDa after endoglycosidase F treatment of renal brush border membranes (Mosckovitz et al., 1993; Estévez, 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 α catalytic subunits of the Na^+/K^+ ATPase by the expression of foreign β subunits of the Na^+/K^+ ATPase (Geering et al., 1989). Interestingly, the cell surface antigen 4F2 is a heterodimer (~125 kDa) composed of a heavy chain of 85 kDa (4F2hc, i.e. the homologous protein to rBAT) and a light chain of 40 kDa linked by disulfide bridges (Haynes et al., 1981; Hemler and Strominger, 1982). Unfortunately, this light subunit evi-

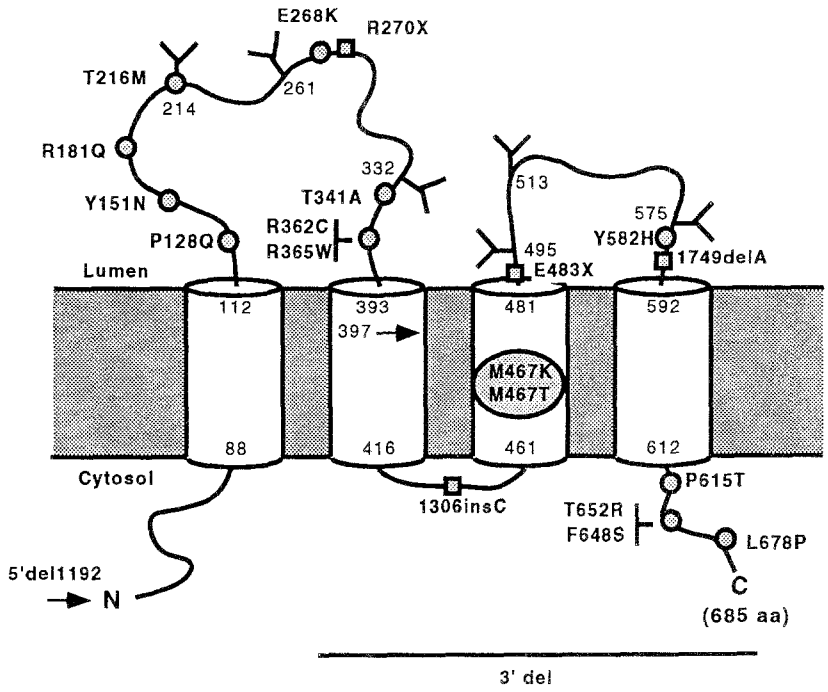

Fig. 3. rBAT protein model with four transmembrane domains as proposed by Tate and coworkers. Three of them would be of amphipatic nature. This model is based on limited proteolysis studies, and the use of different anti-rBAT antibodies in permeable cells (Mosckovitz et al., 1994). Cystinuria-specific 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 labeling 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 (125 kDa) of a "heavy chain" (90 kDa) linked by disulfide bridges to a putative "light chain" of 40–50 kDa. 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; ~2.3 kb and ~4 kb 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 ~5 kb 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

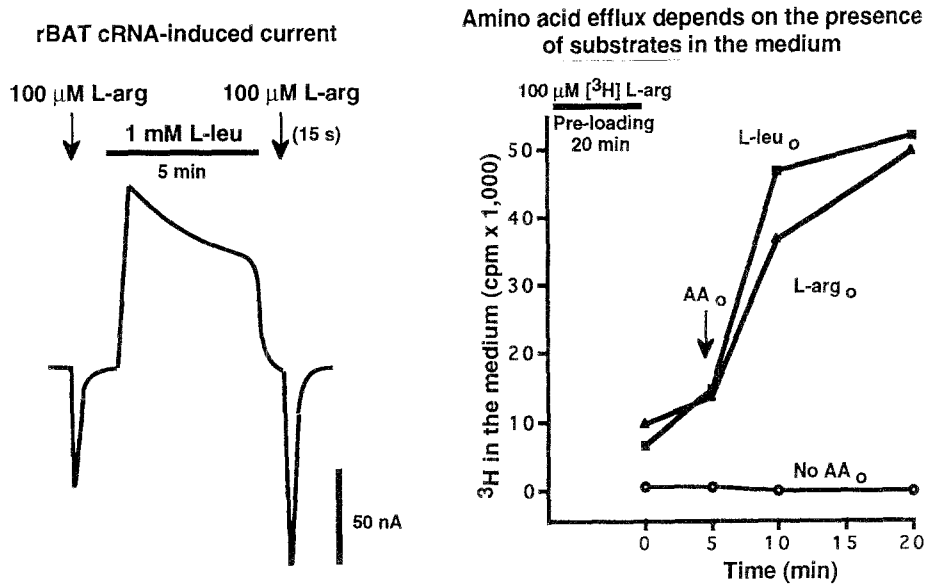

Fig. 4. The transport activity induced by rBAT cRNA in *Xenopus* oocytes is that of an obligatory amino acid exchanger, with substrate specificity similar to the $b^{0,+}$ system. rBAT cRNA expression (1 ng per oocyte) induces an influx current of positive charge when there is arginine in the medium (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., 1996). 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., 1996)

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., 1996). 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., 1996). 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., 1996). 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 the 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).

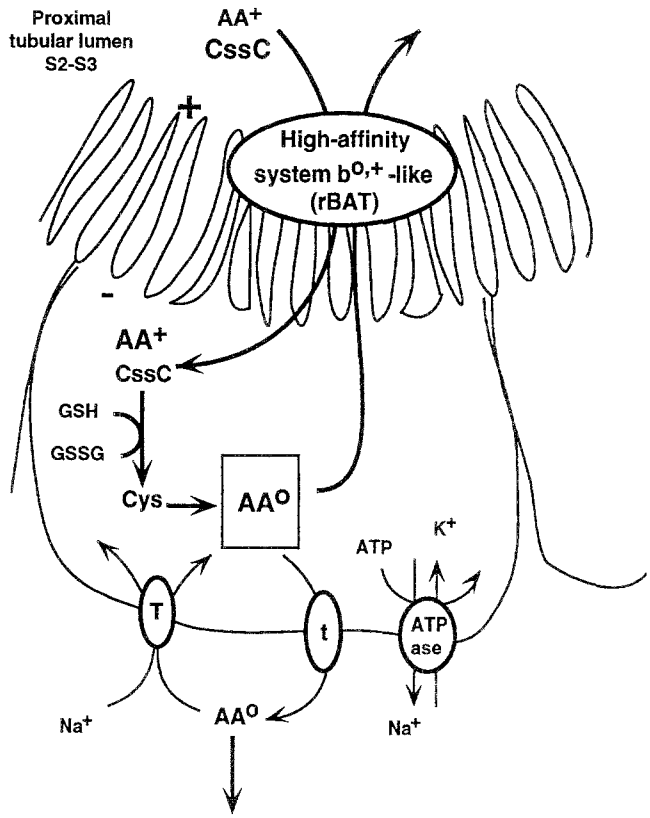

Fig. 5. Model proposing the role of the transport system rBAT/$b^{0,+}$-like in the reabsorption of cystine and dibasic amino acids in the kidney. The rBAT protein would be part of the renal reabsorption system of cystine and dibasic amino acids of high-affinity occurring in the nephron S3 segment. Thus, rBAT would be responsible for 15–20% of the tubular cystine reabsorption in the kidney, which would be of high-affinity and 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 (Segal 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., 1996). 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-specific 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., 1996). 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	East European
1306insC	7	1	East 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. (1995) 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

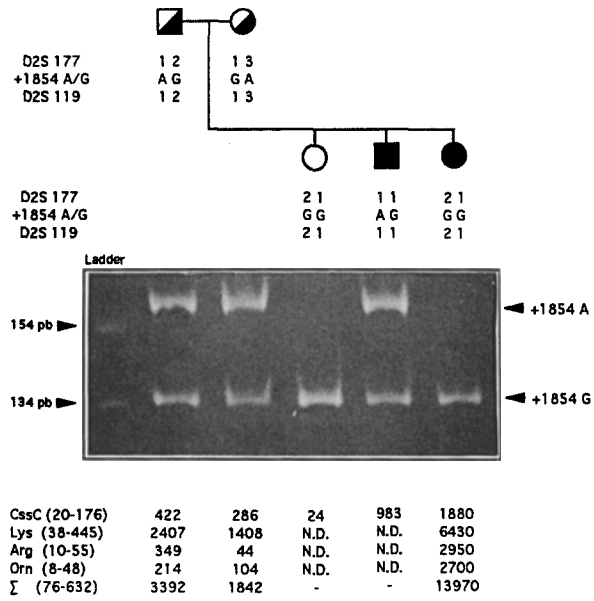

Fig. 7. Family with cystinuria type III/III showing no genetic disequilibrium with the rBAT locus. This family is taken from a study in which genetic heterogeneity linked to cystinuria type III is shown (Calonge et al., 1995b). Filled and half filled symbols represent cystinuric patients and carriers 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., 1996); 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 solubility 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 itself, 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 reabsorption 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 us 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 mechanisms 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.

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