Cloning, functional expression and dietary regulation of the mouse neutral and basic amino acid transporter (NBAT)

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The Na^+-independent dibasic and neutral amino acid transporter NBAT is among the least hydrophobic of mammalian amino acid transporters. The transporter contains one to four transmembrane domains and induces amino acid transport activity via a $b^{0,+}$-like system when expressed in *Xenopus* oocytes. However, the physiological role of NBAT remains unclear. Complementary DNA clones encoding mouse NBAT have now been isolated. The expression of mouse NBAT in *Xenopus* oocytes also induced an obligatory amino acid exchange activity similar to that of the $b^{0,+}$-like system. The amount of NBAT mRNA in mouse kidney increased during postnatal development, consistent with the increase in renal cystine and dibasic transport activity. Dietary aspartate induced a marked increase in cystine transport via the $b^{0,+}$ system in mouse ileum. A high-aspartate diet also increased the amount of NBAT mRNA in mouse ileum. In the ileum of mice fed on the aspartate diet, the extent of cystine transport was further increased by preloading brush border membrane vesicles with lysine. Hybrid depletion of NBAT mRNA from ileal polyadenylated RNA revealed that the increase in cystine transport activity induced by the high-aspartate diet, as measured in *Xenopus* oocytes, was attributable to NBAT. These results demonstrate that mouse NBAT has an important role in cystine transport.

INTRODUCTION

Movement of amino acids across the plasma membrane of animal cells is mediated by both Na^+-independent and Na^+-dependent transport systems. A Na^+-independent neutral basic amino acid transporter (previously named rBAT, D2 and NTAA, but which we refer to here as NBAT) was the first amino acid transporter to be isolated by expression cloning from rats, rabbits and humans [1–5]. Amino acid transport activity of the recombinant protein in *Xenopus* oocytes is similar to that of the $b^{0,+}$-like amino acid transport system, a Na^+-independent carrier that prefers neutral and dibasic amino acids and was initially detected in mouse blastocysts [6]. NBAT is located in the brush border membranes of renal proximal tubules and intestinal epithelial cells [7–9], and defects in this protein have been associated with cystinuria in humans, an autosomal recessive disease characterized by excessive urinary excretion of cystine and the dibasic amino acids arginine, lysine and ornithine [10,11]. DNA sequence analysis suggests that NBAT is a type II membrane glycoprotein with an intracytoplasmic N-terminus and an extracellular C-terminus [1–3]. Thus the putative structure of NBAT suggests that it is not itself a transporter; rather, it might associate with the actual transporter protein [1–3].

The transport rates of most nutrients in the small intestine are regulated by substrate concentrations in the intestinal lumen [12]. In general, the presence of large amounts of protein or amino acids in the diet increases the intestinal transport of amino acids in mice [12]. We have now cloned mouse NBAT cDNA, characterized the functional properties of the encoded protein, investigated developmental changes in the abundance of NBAT mRNA in the kidney and examined the role of NBAT in dietary adaptation in the small intestine.

EXPERIMENTAL

Materials

L-[^{3}H]Arginine (70 Ci/mmol), L-[^{14}C]leucine (300 mCi/mmol) and L-[^{35}S]cystine (300 Ci/mmol) were obtained from DuPont–NEN. Oligonucleotides were synthesized on an Applied Biosystems model 380B DNA synthesizer. Kits for cDNA synthesis were purchased from Pharmacia Biotech; oligo(dT)-cellulose from Becton Dickinson; T7 RNA polymerase from Promega; T4 DNA ligase and restriction endonucleases from Boehringer Mannheim; *in vitro* transcription kits from Ambion; Sequi Therm cycle sequencing kits from AR Brown (Tokyo, Japan); pBluescript SK+ from Stratagene. Male C57BL/6J mice (body weight approx. 50 g) were from SLC (Shizuoka, Japan), and *Xenopus laevis* females from Hamamatsu Jikken (Shizuoka, Japan).

Cloning of mouse NBAT cDNA

Total RNA was extracted from mouse kidney and poly(A)$^+$ RNA was purified by affinity chromatography on oligo(dT)–cellulose columns as previously described [13]. A cDNA library in the vector λgt10 (5×10^5 independent recombinants) was constructed from 5 μg of poly(A)$^+$ RNA with the use of an oligo(dT)-primed cDNA synthesis kit. Plaques were screened by hybridization under high-stringency conditions with ^{32}P-labelled human NBAT cDNA [14]. Six positive clones were isolated, one of which was subcloned into the *Eco*RI site of pBluescript II SK+ and characterized by restriction mapping with *Hin*cII, *Apa*I, *Bam* HI and *Sac*I. Both strands of the cDNA inserts were sequenced with a DNA sequencing kit.

Abbreviations used: BBMV, brush border membrane vesicle; NBAT, neutral and basic amino acid transporter.

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The nucleotide sequence data reported here will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number D88533.

Northern blot analysis

Total RNA from various mouse tissues was denatured, subjected to electrophoresis on a 1.2% (w/v) agarose gel containing formaldehyde, transferred to a nylon membrane and hybridized with ^{32}P-labelled probes; mouse NBAT cDNA, mouse CAT1 cDNA [15] or human 4F2hc cDNA [16] was used as probes.

Transcription and transport assay *in vitro*

Mouse NBAT cDNA was linearized by digestion with *Not*I and transcribed into cRNA with T7 RNA polymerase (Promega, Madison, WI, U.S.A.) [17,18]. Small clumps of oocytes isolated from *X. laevis* females were treated twice for 45 min with 2 mg/ml collagenase in Ca^{2+}-free solution [OR II solution: 82.5 mM NaCl/2 mM KCl/1 mM $MgCl_2$/10 mM Hepes/Tris (pH 7.5)] to remove the follicular layer. After extensive washing, first with OR II solution and then with modified Barth's solution [88 mM NaCl/1 mM KCl/0.82 mM $MgSO_4$/0.4 mM $CaCl_2$/0.33 mM $Ca(NO_3)_2$/2.4 mM $NaHCO_3$/10 mM Hepes/Tris (pH 7.5)], the oocytes were maintained in modified Barth's solution overnight at 18 °C. Healthy stage V oocytes were then injected with 15 ng of NBAT cRNA or 50 ng of poly(A)$^+$ RNA in 50 nl of water with the use of a digital microdispenser; amino acid transport activity was measured after incubation at 18 °C for 5 days. Oocytes were washed for 30 s in solution A [100 mM choline chloride/2 mM KCl/1 mM $CaCl_2$/1 mm $MgCl_2$/10 mM Hepes/Tris (pH 7.5)], after which uptake assays were performed at 20–23 °C for 30 min with six to eight oocytes per experimental group in solution A containing 50 μM L-[^{3}H]arginine, L-[^{14}C]leucine and L-[^{35}S]cystine [14]. The oocytes were then washed three times with ice-cold solution A containing each amino acid at 5 mM. Finally, each oocyte was transferred to a scintillation vial and dissolved in 0.2 ml of 10% (w/v) SDS, and the associated radioactivity was determined by liquid-scintillation spectroscopy after the addition of 5 ml of scintillation fluid.

For the measurement of efflux rates [19], 3 days after injection of the NBAT cRNA (5 ng) groups of six to nine oocytes were incubated at 20–23 °C for 30 min in 200 μl of solution A containing 50 μM L-[^{3}H]arginine. The oocytes were then washed four times with solution A at 20–23 °C, after which the efflux of [^{3}H]arginine was measured by incubation of oocytes at 20–23 °C in 400 μl of solution A in the absence or presence of various L-amino acids. Efflux measurements in the presence of cystine were performed in the additional presence of 10 mM diamide to prevent L-cystine reduction; diamide alone had no effect on [^{3}H]arginine efflux. The amount of radioactivity in a portion of the incubation medium was determined at various times, and efflux rates were corrected for the amount of radioactivity present at zero time. Efflux rates are expressed as the radioactivity (10^3 c.p.m.) appearing in the medium per unit time (5 min) per group of six to nine oocytes.

Feeding mice with diets containing single amino acids

Male C57BL/6J mice (body weight $\sim$ 50 g) were housed in wire-bottomed cages in an animal room maintained at 22 °C with a 12 h light period (08:00 h to 20:00 h). They were provided with a protein-free diet (containing 55% glucose, 30% fat, 9% cellulose, 4% mineral mixture and 1% vitamins) only during a 2 h feeding period (11:00 h to 13:00 h) for 2 weeks and then with test diets for 4 days [20]. The test diets consisted of 5% single amino acid, 4% mineral mixture, 1% vitamins, 9% cellulose, 50% carbohydrate and 30% fat. Control animals continued to receive a protein-free diet for 4 days. The normal diet consisted of 20% casein, 4% mineral mixture, 1% vitamins, 9% cellulose, 50% carbohydrate and 15% fat. On day 5, the animals (final body weight 55–58 g) were killed at 11:00 h, and the entire small intestine was removed quickly and washed with saline. The ileum was used for RNA isolation or preparation of brush border membrane vesicles (BBMVs).

Measurement of amino acid transport in intestinal BBMVs

BBMVs were prepared from mouse ileum by the Ca^{2+} precipitation method, as previously described [21]. Membrane purity was assessed by measuring leucine aminopeptidase, alkaline phosphatase and sucrase activities [21]. Uptake of L-[^{35}S]cystine was measured by a rapid filtration technique [21]. Incubation at 25 °C was initiated by the addition of 10 μl of vesicle suspension to 100 μl of a solution containing 100 mM choline chloride, 100 mM mannitol, 20 mM Hepes/Tris (pH 7.4) and 50 μM L-[^{35}S]cystine. Transport was terminated by rapid dilution of the reaction mixture with 3 ml of ice-cold stop solution [100 mM choline chloride/100 mM mannitol/20 mM Hepes/Tris (pH 7.4)/0.1 mM L-arginine/20 mM $MgSO_4$]. The reaction mixture was then immediately transferred to a pre-moistened filter (0.45 μm) maintained under vacuum.

The effect of lysine efflux on the uptake of [^{35}S]cystine was examined by first loading BBMVs with lysine [22]. Vesicles were incubated for 30 min at 25 °C in transport solution (or in transport solution in which choline chloride had been replaced with NaCl) containing 1 mM unlabelled L-lysine, after which 10 μl of this preincubation suspension was added to 90 μl of transport solution containing 50 μM [^{35}S]cystine, and uptake of the latter was measured [22].

Hybrid depletion of NBAT mRNA from mouse poly(A)$^+$ RNA

Hybrid depletion experiments were performed as previously described [17,18]. Poly(A)$^+$ RNA (1 mg/ml) from the mouse ileum was denatured at 65 °C for 5 min in a solution containing 50 mM NaCl and 40 μM of either mouse NBAT sense (5′-GTGTGTATGGACACTCCAGCTGGCAC-3′) or anti-sense (5′-GTGCCAGCTGGAGTGTCCATACACAC-3′) oligonucleotides, corresponding to the mouse NBAT mRNA sequence from nt 767–792 relative to the translation start site. The mixture was then further incubated at 42 °C for 30 min before the injection of 50 nl into *X. laevis* oocytes. After incubation of the injected oocytes at 18 °C for 5 days in modified Barth's solution, uptake of L-[^{35}S]cystine was measured as described above.

Statistical analysis

Data are presented as means $\pm$ S.E.M. Significance of differences between groups was assessed by one-way analysis of variance followed by the Student–Newman–Keuls test. A P value of < 0.05 was considered statistically significant.

RESULTS

Cloning of mouse NBAT cDNA

We isolated six mouse kidney cDNA clones that hybridized with human NBAT cDNA. One of these clones, B-67, contained a full-length insert of 2287 bp, similar to the size of the predominant NBAT mRNA in mouse kidney as estimated by Northern blot analysis (see below). The first ATG codon of the cDNA is located within a consensus initiation sequence. The open reading frame continues to the first stop codon (TAG) at position 2103 and encodes a 685-residue protein, with a calculated molecular mass of 78372 Da, that shares 78.0%, 91.0% and 78.9% identity

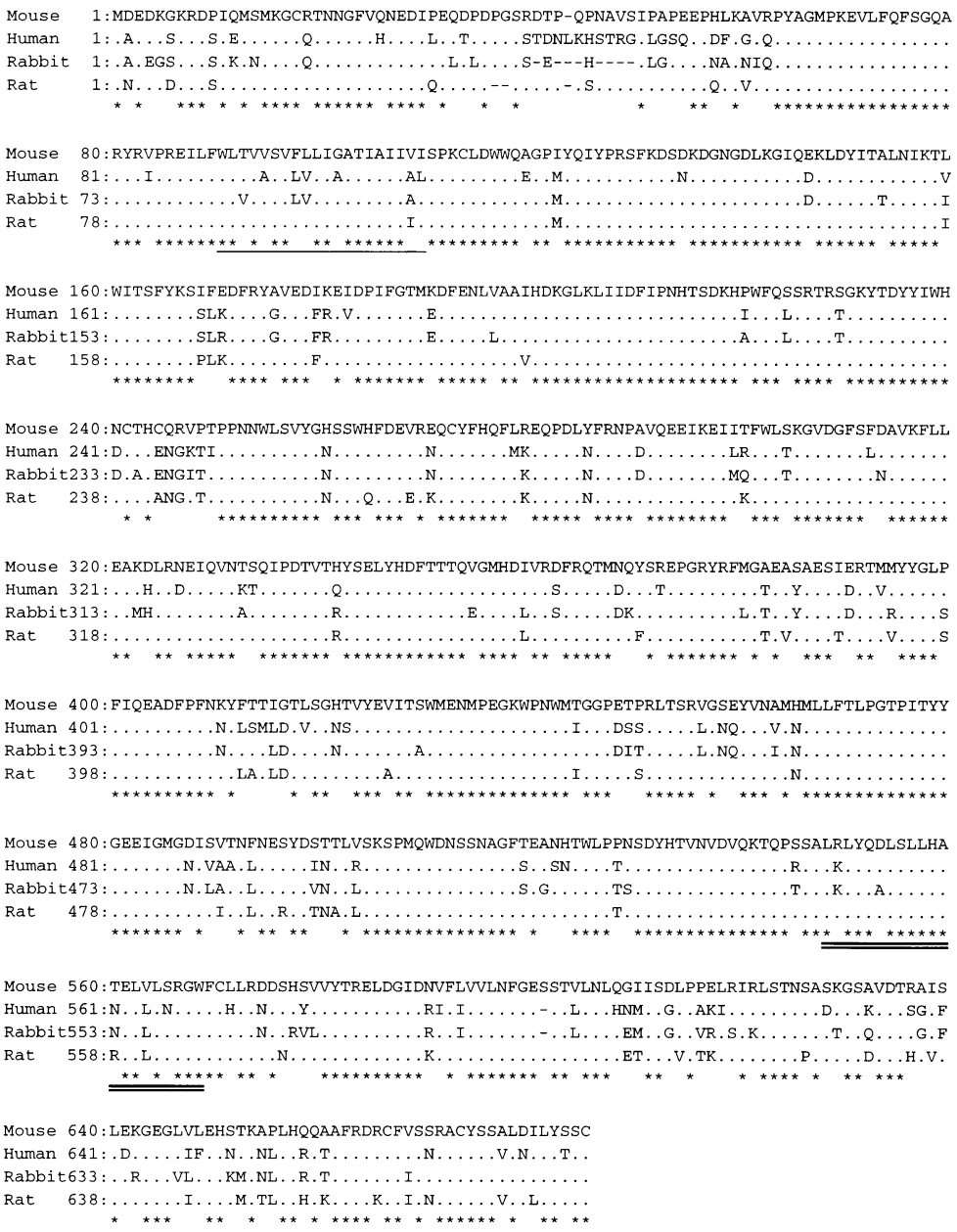

Figure 1 Comparison of amino acid sequences of mouse, human, rabbit and rat NBAT

Amino acids are indicated by single-letter abbreviations. Residues identical to those in mouse NBAT are indicated by dots; residues identical in all four sequences are indicated by asterisks. Hyphens represent gaps introduced to optimize alignment. The putative transmembrane domain and leucine zipper motif are marked by single and double underlines respectively.

with rabbit, rat and human NBAT respectively (Figure 1). Hydropathy analysis of the predicted amino acid sequence detected a single putative transmembrane domain. A leucine zipper motif is conserved in the C-terminal region of NBAT in the four species. Putative N-glycosylation sites are present at residues 123, 234, 345 and 349 in the extracellular domain.

Properties of NBAT-mediated amino acid transport

Xenopus oocytes were injected with cRNA synthesized from mouse NBAT cDNA, and amino acid transport activity was determined. Sodium-independent uptake of L-arginine, L-leucine or L-cystine was increased in oocytes injected with NBAT cRNA compared with that in water-injected control oocytes (Table 1). Previous studies have suggested that the $b^{0,+}$-like amino acid transport system associated with NBAT expression in oocytes is an amino acid exchanger [23–25]. The presence of dibasic or neutral amino acids (including L-cystine), which are substrates for the $b^{0,+}$-like system, in the external medium increased the efflux of L-arginine from oocytes injected with NBAT cRNA (Table 2) but not from uninjected oocytes (results not shown). L-Proline and L-glutamate, which are not substrates for the $b^{0,+}$-like transport system, did not increase the efflux of [^{3}H]arginine from oocytes injected with NBAT cRNA (results not shown).

Table 1 Transport of amino acids in *Xenopus* oocytes injected with mouse NBAT cRNA

Five days after injection with 50 nl of water or 50 nl of water containing 15 ng of mouse NBAT cRNA, oocytes were assayed for uptake of L-[^{3}H]arginine, L-[^{14}C]leucine or L-[^{35}S]cystine in the absence (100 mM choline chloride) or presence (100 mM NaCl) of Na^+. Uptake incubations were performed for 30 min at 20–23 °C, after which the oocytes were washed and the amount of radioactivity associated with each was determined. Results are means ± S.E.M. for six to eight oocytes from a representative experiment.

		Uptake (pmol/min per oocyte)	
Amino acid	Injection with	$-Na^+$	$+Na^+$
L-Arginine	Water	0.24 ± 0.11	0.48 ± 0.14
	NBAT	13.4 ± 2.3	16.3 ± 1.5
L-Leucine	Water	0.14 ± 0.05	0.39 ± 0.12
	NBAT	11.1 ± 2.6	14.8 ± 3.5
L-Cystine	Water	0.10 ± 0.04	0.16 ± 0.04
	NBAT	6.6 ± 1.5	9.0 ± 1.2

Table 2 Effects of various amino acids on the efflux of L-[^{3}H]arginine from *Xenopus* oocytes expressing mouse NBAT

Oocytes were injected with 15 ng of NBAT cRNA and, after 3 days, the efflux of L-[^{3}H]arginine was measured at 20–23 °C in choline medium in the absence (None) or presence of 500 μM L-arginine, L-lysine, L-leucine or 200 μM L-cystine (plus 10 mM diamide). Efflux rates, expressed as radioactivity appearing in the incubation medium per 5 min per group of nine oocytes, are means ± S.E.M. for triplicate determinations from a representative experiment. $^*P < 0.05$, $^{**}P < 0.01$ compared with the respective None group.

Amino acid	L-[^{3}H]Arginine efflux (10^{-3} c.p.m./5 min per nine oocytes)
None	20 ± 14
L-Arginine	228 ± 43**
None	46 ± 19
L-Lysine	195 ± 45**
None	15 ± 7
L-Leucine	142 ± 36**
None	34 ± 18
L-Cystine	76 ± 16*

Distribution of NBAT mRNA in mouse tissues

Northern analysis detected four prominent NBAT transcripts of approx. 2.4, 4.2, 5.4 and 8.0 kb in the kidney; all except the 5.4 kb species were also observed in the small intestine (Figure 2). Only a 4.2 kb NBAT mRNA was apparent in the brain (results not shown). The 2.4 kb transcript was the predominant form in the kidney and small intestine and was also prominent in the liver (Figure 2). No NBAT transcripts were detected in mouse heart, colon, spleen or muscle (results not shown).

Developmental changes in the abundance of NBAT mRNA in mouse kidney

NBAT mRNA was not detected in the kidney of 2-day-old mice. It was first apparent in 4-day-old animals, after which it gradually increased in abundance to adult levels (Figure 3).

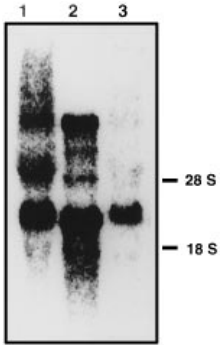

Figure 2 Tissue distribution of NBAT mRNA

Total RNA (approx. 20 μg) from mouse ileum (lane 1), kidney (lane 2) and liver (lane 3) was resolved on a 1.2% (w/v) agarose gel containing formaldehyde, then transferred to a nylon membrane and hybridized with a ^{32}P-labelled mouse NBAT cDNA probe. Hybridizing species were detected by autoradiography. The positions of 28 S and 18 S rRNA species are indicated.

Relation between intestinal cystine transport and abundance of NBAT mRNA

To study the induction of amino acid transport in the mouse small intestine, we fed mice on a protein-free diet for 2 weeks and then a diet containing a single amino acid (lysine, arginine, alanine, glycine, aspartate or glutamate) for 4 days before measuring cystine transport activity in BBMVs isolated from the ileum. Cystine transport in BBMVs from rats fed on a protein-free diet for 2 weeks did not differ from that in vesicles prepared from mice fed on a normal diet (results not shown). Of the amino acids tested, only aspartate in the diet significantly increased cystine uptake in ileal BBMVs (Table 3). Cystine transport was inhibited by the presence of 5 mM alanine, leucine, arginine or lysine in the uptake assay (results not shown), suggesting that it was mediated by the $b^{0,+}$ system. The increase in cystine transport induced by dietary aspartate was due to an increase in V_{max} rather than to a decrease in K_m (Table 3).

Northern blot analysis also revealed that the amount of the 2.4 kb NBAT mRNA in the ileum of mice fed on the aspartate diet for 4 days was approx. 2.8 times that in mice fed on the control protein-free diet (Figure 4). In contrast, the abundance of CAT1 and 4F2hc mRNA species, which encode y^+- and y^+L-like transporters respectively in the small intestine, did not differ between mice fed on the aspartate or protein-free diet.

Effect of lysine efflux on uptake of cystine by ileal BBMVs isolated from mice fed on an aspartate diet

Because NBAT is thought to mediate the exchange of a neutral amino acid for a dibasic amino acid [23–25], we also measured the influx of L-cystine into BBMVs that had been preloaded with L-lysine. In the BBMVs isolated from the ileum of mice fed on the aspartate diet, the presence of intravesicular lysine increased the initial uptake of extravesicular cystine in the presence or absence of a Na^+ gradient, although the effect was greater in the presence of such a gradient (Figure 5). However, the stimulation of the exchange activity was not observed in the BBMVs isolated from the mice fed on the protein-free diet (results not shown).

Amino acid inhibition and hybrid depletion analyses of the role of NBAT in dietary adaptation of intestinal cystine transport activity

To investigate further whether the increase in ileal cystine uptake in mice fed on an aspartate diet is mediated by the increase in the

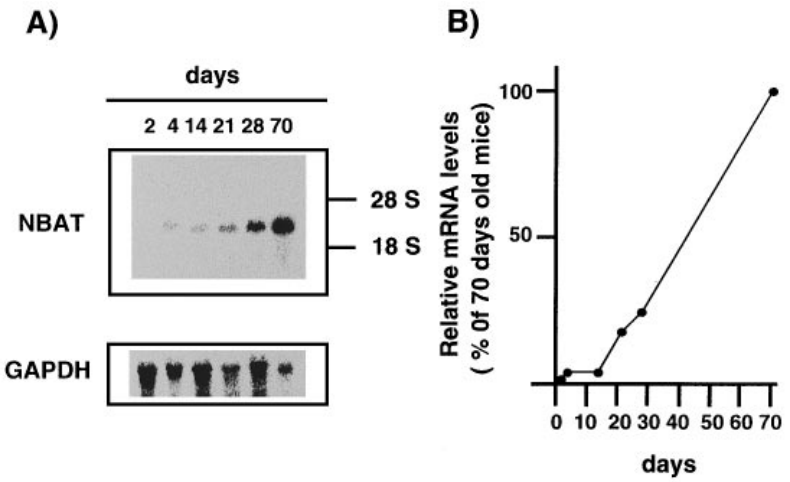

Figure 3 Developmental changes in the abundance of NBAT mRNA in mouse kidney

(**A**) Total RNA (approx. 10 μg) from the kidneys of developing mice was subjected to Northern blot analysis with ^{32}P-labelled mouse NBAT cDNA (upper panel) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (lower panel) probes. (**B**) The relative abundances of mouse NBAT mRNA species were determined by image analysis with a Fuji BAS 2000 system and were corrected for differences in RNA loading as revealed by differences in the intensity of the GAPDH mRNA band. Results are expressed relative to the maximum NBAT mRNA abundance observed (in 70-day-old mice) and are representative of two similar experiments.

Table 3 Effects of dietary amino acids on L-cystine transport in mouse ileal BBMVs

Animals were fed on a protein-free diet for 2 weeks and then either maintained on this diet or switched to a diet containing a single amino acid at 5% for 4 days. On the fifth day the mice were killed and cystine transport activity in BBMVs prepared from the ileum was determined in the absence of Na^+. The V_{max} of transport (pmol of cystine/15 s per mg of protein) and the K_m for cystine were calculated. Results are means $\pm$ S.E.M. for four mice. $^{**}P < 0.05$ compared with protein-free diet.

Diet	V_{max} (pmol/15 s per mg of protein)	K_m (mM)
Normal	144 $\pm$ 48	0.12 $\pm$ 0.03
Protein-free	128 $\pm$ 32	0.11 $\pm$ 0.02
Glycine	151 $\pm$ 31	0.13 $\pm$ 0.02
Alanine	134 $\pm$ 41	0.14 $\pm$ 0.03
Lysine	157 $\pm$ 25	0.11 $\pm$ 0.02
Arginine	169 $\pm$ 18	0.14 $\pm$ 0.04
Aspartate	228 $\pm$ 34**	0.12 $\pm$ 0.03
Glutamate	127 $\pm$ 32	0.11 $\pm$ 0.02

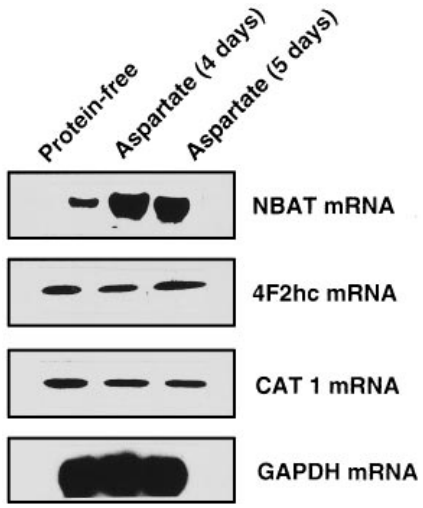

Figure 4 Effect of an aspartate diet on the abundance of NBAT mRNA in the mouse small intestine

Mice were fed on a protein-free diet for 2 weeks and then maintained on this diet for 4 days or switched to an aspartate diet for 4 days. Animals were killed on the day after the test diet period, and ileal total RNA was subjected to Northern blot analysis with probes specific for NBAT, 4F2hc, CAT1 or GAPDH mRNA as indicated. Results are representative of three similar experiments.

abundance of NBAT mRNA, we measured cystine transport activity in *Xenopus* oocytes injected with poly(A)$^+$ RNA from the ileum of mice fed on the aspartate or protein-free diet. The extent of cystine uptake at 30 min in oocytes injected with RNA from mice fed on the aspartate diet was approx. 2.5 times that apparent in oocytes injected with RNA from mice fed on the protein-free diet (Figure 6).

Cystine transport in oocytes injected with poly(A)$^+$ RNA from the ileum of mice fed on the aspartate diet was inhibited by L-arginine and L-leucine, but not by L-homoserine (a substrate for the system y^+) or methylaminoisobutyric acid (a specific substrate for the Na^+-dependent neutral amino acid transport system A), suggesting that cystine uptake is probably mediated by the $b^{0,+}$ system (Table 4). Moreover, dietary aspartate did not stimulate the activity of other amino acid transport systems in the small intestine (results not shown).

Finally, hybrid depletion analysis with an NBAT anti-sense oligonucleotide, but not the corresponding sense oligonucleotide, completely abolished the aspartate-diet-induced increase in oocytes microinjected with ileal poly(A)$^+$ RNA (Table 5).

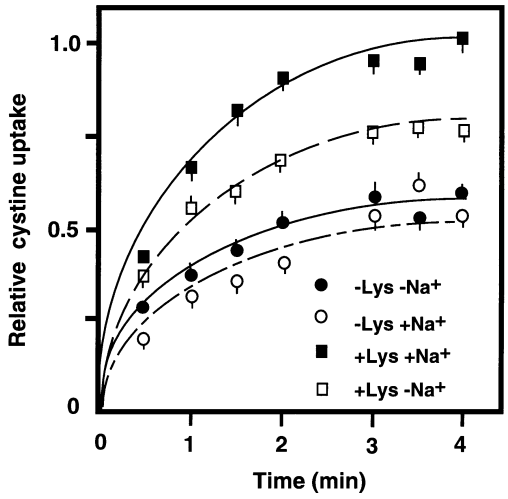

Figure 5 Effect of L-lysine efflux on uptake of L-cystine by ileal BBMVs from mice fed on an aspartate diet

Mice were fed on a protein-free diet for 2 weeks and then switched to an aspartate diet for 4 days. Animals were killed on the next day and ileal BBMVs were isolated. The vesicles were incubated for 30 min in the absence or presence of 1 mM L-lysine and in the presence of either 100 mM choline chloride or 100 mM NaCl. Uptake of L-[^{35}S]cystine (50μM) was then measured in the absence or presence of extravesicular Na^+. Uptake was expressed relative to that observed at 5 min with BBMVs, from mouse fed on the aspartate diet, that were incubated with lysine in the presence of NaCl. Results are means $\pm$ S.E.M. for five mice.

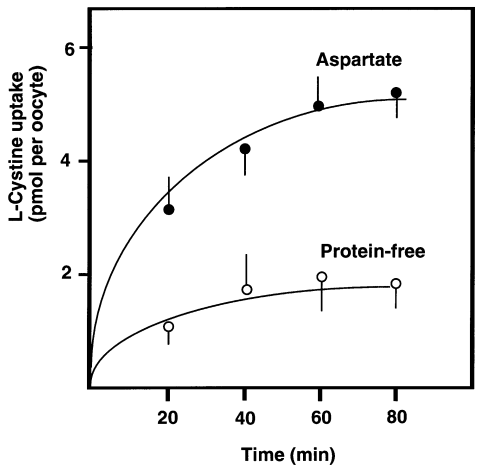

Figure 6 Time course of uptake of L-[^{35}S]cystine in *Xenopus* oocytes injected with poly(A)$^+$ RNA from the ileum of mice fed on an aspartate diet or a protein-free diet

Xenopus oocytes were injected with 50 ng of poly(A)$^+$ RNA from the ileum of mice fed on a protein-free or aspartate diet for 4 days. The time course of L-cystine uptake was measured after 5 days. Results have been corrected for the amount of uptake observed in water-injected oocytes and are means $\pm$ S.E.M. for five oocytes in a representative experiment.

Table 4 Effects of various amino acids on the uptake of L-cystine by *Xenopus* oocytes injected with ileal poly(A)$^+$ RNA from mice fed on an aspartate diet

Oocytes were injected with 50 ng of ileal poly(A)$^+$ RNA isolated from mice fed on the aspartate diet for 4 days. After 5 days the uptake of L-[^{35}S]cystine (50 μM) was measured in the absence or presence of the indicated non-radioactive amino acids at a concentration of 5 mM. Results have been corrected for uptake observed with water-injected oocytes and are means $\pm$ S.E.M. for six oocytes in a representative experiment. $^*P < 0.01$, $^{**}P < 0.001$ compared with control.

Amino acid (5 mM)	L-Cystine uptake (pmol/ 20 min per oocyte)
None	3.62 $\pm$ 0.87
L-Cystine	0.55 $\pm$ 0.11**
L-Arginine	0.54 $\pm$ 0.21**
L-Homoserine	3.39 $\pm$ 0.98
L-Leucine	1.55 $\pm$ 0.41*
Methylaminoisobutyric acid	3.48 $\pm$ 0.69

Table 5 Hybrid depletion analysis of the role of NBAT in the aspartate-diet-induced increase in L-cystine uptake in *Xenopus* oocytes injected with mouse ileal poly(A)$^+$ RNA

Ileal poly(A)$^+$ RNA from mice fed on the aspartate diet for 4 days was incubated with NBAT sense or anti-sense oligonucleotides before injection into *Xenopus* oocytes. Oocytes were also injected with water or untreated poly(A)$^+$ RNA from mice fed on the protein-free or aspartate diet. L-Cystine uptake was measured 5 days after oocyte injection. Results are means $\pm$ S.E.M. for six to eight oocytes. $^*P < 0.01$ compared with (+) sense oligonucleotide.

Injection	L-Cystine uptake (pmol/ 20 min per oocyte)
Water	0.51 $\pm$ 0.14
Protein-free diet poly(A)$^+$ RNA	1.21 $\pm$ 0.36
Aspartate diet poly(A)$^+$ RNA, untreated	3.22 $\pm$ 0.51
(+) Sense oligonucleotide	3.54 $\pm$ 0.42
(+) Anti-sense oligonucleotide	1.15 $\pm$ 0.22*

DISCUSSION

The predicted amino acid sequence of mouse NBAT indicates that it is a type II membrane glycoprotein, as apparently are human, rat and rabbit NBAT proteins [1–5]. The abundance of mouse NBAT mRNA is high in kidney and small intestine, tissues in which the $b^{0,+}$ amino acid transport system is present [6–9]. Transcripts of 2.4, 4.2, 5.4 or 8.0 kb hybridized to NBAT cDNA in mouse kidney and small intestine; given that the relative intensities of these bands do not change with hybridization stringency (results not shown), they might result from the use of different polyadenylation sites. These observations are consistent with those obtained with the NBAT mRNA species in rabbit, rat and human [1–5]. The developmental increase in the amount of NBAT mRNA in mouse kidney paralleled the increase in cystine transport activity also apparent in this tissue [26].

Cystinuria-specific mutations in the NBAT gene have been detected only in individuals with type I cystinuria [27,28]. Type I homozygotes do not show increases in the plasma concentration of cystine and arginine after oral administration of these amino acids, suggesting that NBAT is required for the transport of cystine and dibasic amino acids in the brush border membrane of the small intestine. Magagnin et al. [29] showed that hybrid depletion of NBAT mRNA from rabbit jejunum poly(A)$^+$ RNA virtually abolished RNA-induced L-cystine uptake in *Xenopus* oocytes. However, the functional role of NBAT in human, rat

and mouse has remained unknown, in part because of low levels of cystine transport activity induced in oocytes by the injection of mRNA.

Stein et al. [20] showed that, in mice, dietary lysine typically stimulates the uptake of basic amino acids, whereas dietary valine enhances the uptake of neutral amino acids. They also showed, however, that the acidic amino acid aspartate induces its own transport as well as that of lysine. In the present study, a high-aspartate diet significantly increased cystine uptake subsequently measured in ileal BBMVs in the absence of Na^+. The amount of NBAT mRNA in the ileum was also increased 2.8-fold in aspartate-fed mice. These results indicate that NBAT might be associated with a cystine-transporting neutral and dibasic amino acid transport system (the $b^{0,+}$ system). Amino acid inhibition studies suggested that the aspartate-diet-induced increase in cystine transport activity was not mediated by the y^+ system or other amino acid transport systems. Hybrid depletion with CAT1 or 4F2hc anti-sense oligonucleotides, unlike that with an NBAT anti-sense oligonucleotide, did not affect the L-cystine uptake induced by injection of ileal RNA from aspartate-fed mice into *Xenopus* oocytes (results not shown). These observations indicate that NBAT is required for cystine transport in mouse small intestine. In addition, the cellular localization of NBAT in intestinal microvilli is consistent with its role as a transporter of amino acids across epithelial cells in this tissue [9,26].

It is not clear whether aspartate induces the expression of the NBAT gene directly or indirectly through the action of an intermediate messenger such as a hormone. Candidates for such a humoral mediator include gastrin, glucagon, enteroglucagon, cholecystokinin, secretin, insulin, prolactin, adrenaline, prostaglandin E1 and androgenic and oestrogenic steroids [12]. The rat NBAT gene promoter contains putative binding sites for various transcription factors, such as CCAAT-enhancer-binding protein, hepatocyte nuclear factor 1-β and hepatocyte nuclear factor 4 [30], that are abundant in kidney or liver. However, unlike mouse NBAT mRNA, rat, rabbit and human NBAT transcripts were not detected in liver. The regulation of NBAT expression in mice also seems to differ from that in other species in that dietary aspartate did not increase the amount of intestinal NBAT mRNA in rats and rabbits (results not shown). A high-protein diet increased the abundance of mRNA species encoding the intestinal peptide transporter PepT1 and the high-affinity glutamate transporter EAAC1 in mice, but had no effect on that of the mRNAs in rats [31]. In contrast, the amount of NBAT mRNA in the small intestine was markedly increased by a high-protein diet in mice (results not shown).

The microinjection of mouse NBAT cRNA into *Xenopus* oocytes also stimulated neutral and dibasic amino acid transport activity, as demonstrated previously for other species [1–5]. Recent studies indicate that NBAT is a transport- (or exchange-) activating protein [14,32]. We previously demonstrated that a mutant NBAT protein (Δ511–685) stimulates an endogenous y^+-like transport system in *Xenopus* oocytes that is distinct from the $b^{0,+}$ system activated by wild-type NBAT [14]. Ahmed et al. [32] also showed that NBAT stimulates functionally distinct amino acid transporters in *Xenopus* oocytes. Wang and Tate [33] showed that NBAT is associated with a brush border membrane protein of approx. 50 kDa and they proposed that this heterodimer represents the minimal functional unit of the high-affinity cystine transporter in renal and intestinal membranes.

NBAT also stimulates a neutral and dibasic amino acid exchange activity [23–25]. The presence of a neutral amino acid at the *trans* site is required to produce the currents associated with the movement of dibasic amino acids in oocytes and kidney cells expressing NBAT [23–25]. This observation suggests an obligatory exchange mechanism for amino acid transport induced by NBAT. The dibasic-inwards, neutral-outwards direction of exchange is predominant in oocytes and is consistent with the observation that hyperexcretion of neutral amino acids does not occur in cystinuric patients [34]. We also observed that NBAT stimulates an obligatory exchange activity in *Xenopus* oocytes as well as in BBMVs isolated from mice fed on an aspartate diet. McNamara et al. [22] demonstrated that cystine uptake in the presence of extracellular Na^+ was markedly increased in renal BBMVs that had been preloaded with L-lysine. In contrast, in the present study the obligatory exchange activity of NBAT in *Xenopus* oocytes was independent of extracellular Na^+, whereas that in intestinal BBMVs was partly dependent on extracellular Na^+. This partial Na^+ dependence might result from BBMVs isolated from mouse small intestine containing a y^+L-like transport system associated with 4F2hc. Functional asymmetry of amino acid exchange has been demonstrated for the y^+L-like system expressed in oocytes, with movement of dibasic amino acids inwards and neutral amino acids outwards in the presence of extracellular Na^+ [19]. The difference between the exchange activity of oocytes expressing mouse NBAT and BBMVs might also result from the presence of CAT1 in BBMVs.

In conclusion we have shown that mouse NBAT has an important role in cystine transport in the kidney and small intestine.

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