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Cloning and Functional Expression of a Human Na^+ and Cl^--dependent Neutral and Cationic Amino Acid Transporter B^{0+}*

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A Na^+-dependent neutral and cationic amino acid transport system (B^{0+}) plays an important role in many cells and tissues; however, the molecular basis for this transport system is still unknown. To identify new transporters, the expressed sequence tag database was queried, and cDNA fragments with sequence similarity to the Na^+/Cl^--dependent neurotransmitter transporter family were identified. Based on these sequences, rapid amplification of cDNA ends of human mammary gland cDNA was used to obtain a cDNA of 4.5 kilobases (kb). The open reading frame encodes a 642-amino acid protein named amino acid transporter B^{0+}. Human ATB^{0+} ($hATB^{0+}$) is a novel member of the Na^+/Cl^--dependent neurotransmitter transporter family with the highest sequence similarity to the glycine and proline transporters. Northern blot analysis identified transcripts of ~4.5 kb and ~2 kb in the lung. Another tissue survey suggests expression in the trachea, salivary gland, mammary gland, stomach, and pituitary gland. Electrophysiology and radiolabeled amino acid uptake measurements were used to functionally characterize the transporter expressed in *Xenopus* oocytes. $hATB^{0+}$ was found to transport both neutral and cationic amino acids, with the highest affinity for hydrophobic amino acids and the lowest affinity for proline. Amino acid transport was Na^+ and Cl^--dependent and was attenuated in the presence of 2-aminobicyclo-[2.2.1]-heptane-2-carboxylic acid, a system B^{0+} inhibitor. These characteristics are consistent with system B^{0+} amino acid transport. Thus, $hATB^{0+}$ is the first cloned B^{0+} amino acid transporter.

Amino acids are involved in biosynthetic pathways, act as neurotransmitters, and are essential for metabolic processes. Amino acids do not permeate cell membranes and therefore require specialized transport proteins in order to cross the plasma membrane (1). Transporters are classified based on sequence similarity, amino acid substrate specificity, and ion dependence. Ion-independent transporters carry amino acids according to their electrochemical gradient, whereas ion-coupled transporters use ion motive force to concentrate amino acids inside the cell (2).

Mammalian plasma membrane amino acid transporters have been functionally classified into two groups based on their Na^+-independent or Na^+-dependent mechanism of action (3, 4). Two gene families encode transporters that mediate Na^+-independent amino acid transport. One such family, the cationic amino acid transporters, CAT1–CAT4, carries lysine, arginine, and histidine and possesses much lower affinity for other amino acids (5). Na^+-independent amino acid transport is also induced by another family of proteins, which include 4f2hc (6, 7) and rBAT (8, 9). These proteins are not transporters themselves but rather have recently been shown to form heteromultimers with other proteins, y+LAT (10, 11), LAT1 (12), or xCT (13), which show homology to the CAT family. The amino acid substrate specificity of these complexes depends on the specific subunit composition (10–13).

Na^+-dependent transporters utilize the electrochemical gradients of Na^+ and other ions to actively transport amino acids. There are two gene families that encode Na^+-dependent amino acid transporters. One Na^+-dependent transporter family includes the excitatory amino acid transporters that transport glutamate and aspartate, EAAT1–5, the transporters for alanine, serine, and cysteine, ASCT1 and ASCT2, and the neutral amino acid transporter $hATB^0$ (14, 15). In addition to cotransport of amino acids and Na^+, members of this family have been reported to cotransport H^+ and countertransport K^+ (16, 17). An additional amino acid transporter family utilizes Cl^- along with Na^+ to transport amino acids and other organic substrates into the cell (18, 19). The Na^+/Cl^--dependent transporter family includes transporters for γ-aminobutyric acid-like substrates (*e.g.* betaine and taurine), monoamines (*e.g.* serotonin and dopamine), and amino acids (*e.g.* glycine and proline) (20).

The recent cloning of transporter genes enables correlation between individual transport proteins and transport systems described in specific cell types or tissues (21). Transport systems for amino acids have been classically characterized based on amino acid specificity, ion dependence, and pharmacological properties (1). One such transport system, designated B^{0+}, is defined by Na^+-dependent transport of both neutral and cationic amino acids (22). System B^{0+} transport has been reported in mouse blastocysts (22), *Xenopus* oocytes (23–26), a human intestinal cell line (27), rabbit small intestine (28), rabbit conjunctiva (29, 30), rat pituitary gland (31), bullfrog lung (32), and human lung (33). The molecular basis of this transport system is yet unknown.

In this study, we report the cloning and functional expression of a novel human amino acid transporter, $hATB^{0+}$.[1] $hATB^{0+}$ is a member of the Na^+/Cl^--dependent neurotransmitter transporter family and transports both neutral and cationic amino acids in a Na^+- and Cl^--dependent manner. Substrate specificity and pharmacology indicate that $hATB^{0+}$ is

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[1] The abbreviations used are as follows: $hATB^{0+}$, human amino acid transporter B^{0+}; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; kb, kilobases; BCH, 2-aminobicyclo-[2.2.1]-heptane-2-carboxylic acid; EST, expressed sequence tag; EC_{50}, half-maximal effective concentration; I_{max}, maximal current response.

the first molecularly characterized system B^{0+} amino acid transporter.

EXPERIMENTAL PROCEDURES

Molecular Cloning—Gene-specific primers were paired with adaptor ligated sequence-specific primers, AP1 and AP2, for rapid amplification of cDNA ends (RACE) (34) using Advantage Polymerase™ (CLONTECH). Primers were originally designed based on GenBank accession number AA526963 and were subsequently designed based on 5′ and 3′ RACE clones and correspond to nucleotides 534–558 and 577–596 for 5′ RACE and nucleotides 430–455 and 1969–1994 for 3′ RACE (Life Technologies, Inc.). For primary 5′ and 3′ RACE, a gene-specific primer was paired with AP1 to amplify mammary Marathon Ready™ cDNA (CLONTECH). This PCR product was diluted 1:500 for secondary RACE reactions using a nested gene-specific primer and AP2. All 5′ and 3′ RACE products were subcloned into pCR2.1-TOPO and transformed into TOP10 *Escherichia coli* using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA). Individual colonies were screened for insert size with PCR using M13 forward and reverse primers. DNA was sequenced at the University's Automated DNA Sequencing Facility on a Model 373A DNA Sequencer using the *Taq* DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Lasergene (DNA Star, Madison, WI) and Basic Local Alignment Search Tool (BLAST) were utilized for sequence analysis (35). To determine genomic structure, the BLAST 2 Sequences program was used to align genomic sequences (GenBank accession numbers AL034411 and Z96810) with the hATB^{0+} cDNA sequence. Protein motifs were identified using ScanProsite (36), and membrane topology was predicted by TMpred (37) and Kyte-Doolittle hydrophobicity analysis.

Northern and Master Blots—The probe was synthesized by PCR amplification of nucleotides 710–1896 of the hATB^{0+} cDNA and labeled by random priming with [^{32}P]dCTP (Amersham Pharmacia Biotech) to a specific activity of approximately 1×10^7 cpm/ml using the Random Prime Labeling Kit (Roche Molecular Biochemicals). After prehybridization for 30 min at 68 °C in ExpressHyb™ solution (CLONTECH), a human Multiple Tissue Northern blot and a human Master blot (CLONTECH) were hybridized with the cDNA probe in ExpressHyb™ for 1 and 6 h, respectively. The Northern blot was washed three times with 2× SSC and 0.05% SDS at room temperature and twice with 0.1× SSC and 0.1% SDS at 50 °C. The Master blot was washed five times in 2× SSC and 1% SDS at 65 °C and twice in 0.1× SSC and 0.5% SDS at 55 °C. Both blots were subsequently exposed to a PhosphorImager screen (Molecular Dynamics) for 24 h or to autoradiography film for 48 h at −70 °C.

Expression in Xenopus Oocytes—To obtain the open reading frame for functional analysis, two primers corresponding to nucleotides 74–105 and 2001–2027 of the hATB^{0+} cDNA sequence were designed. PCR amplification of mammary gland Marathon Ready™ cDNA with these primers yielded a single DNA band of ~1.9 kb that was ligated into pCR2.1-TOPO. For efficient expression in *Xenopus* oocytes, the hATB^{0+} coding sequence was transferred into pKSPA, a modified pBluescript KS+ plasmid (Stratagene, La Jolla, CA) containing $(A)_{30}$, using *Xba*I and *Hin*dIII sites. One clone was subsequently used for all functional studies. The template for cRNA synthesis was prepared by *Not*I digestion of hATB^{0+}-pKSPA or by PCR of hATB^{0+}-pKSPA using M13 forward and reverse primers. cRNA was synthesized *in vitro* with T7 RNA polymerase (mMessage mMachine; Ambion, Austin, TX). *Xenopus* oocytes were surgically removed, treated, and selected as described previously (38, 39). In brief, *Xenopus laevis* (Nasco, Fort Atkinson, WI) were anesthetized with 0.2% Tricaine. Oocytes were removed and treated with 4 mg/ml type 1A collagenase (Sigma) in a Ca^2 -free solution (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES (pH 7.5) with 50 μg/ml gentamycin) for 1 h at room temperature. Oocytes were rinsed with the Ca^{2+}-free solution and then rinsed with modified Ringer's solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.5) supplemented with 0.5 mM pyruvate and 50 μg/ml gentamycin. Stage IV oocytes were selected and injected the following day with 5–10 ng of cRNA or water in a total volume of 50 nl. Oocytes were maintained at 19 °C in Ringer's solution supplemented with 0.5 mM pyruvate and 50 μg/ml gentamycin. Three to seven days after injection, oocytes were used for electrophysiology or uptake experiments. All experiments were performed at room temperature (21 °C).

Electrophysiology—Two electrode voltage clamp experiments were conducted using a GeneClamp 500 amplifier (Axon Instruments, Foster City, CA). Current was measured upon application of increasing concentrations of amino acids ranging from 1 μM to 10 mM for ~15 s and washed for a period of ~30 s in Ringer's solution. In ion dependence experiments, the NaCl concentration of the Ringer's solution was 100 mM, and Na^+ and Cl^- were substituted with equimolar concentrations of *N*-methyl-D-glucamine and gluconate, respectively. All experiments were conducted at a holding potential of −80 mV. Clampex was used to acquire data at 62.5 Hz (pClamp 6; Axon Instruments). After digital filtering at 1 Hz, data were analyzed by Clampfit (pClamp 6; Axon Instruments).

Uptake Experiments—L-[4,5-^{3}H]Leucine (136 Ci/mmol), L-[2,3,4,5-^{3}H]arginine monohydrochloride (71 Ci/mmol), and L-[G-^{3}H]glutamic acid (136 Ci/mmol) (Amersham Pharmacia Biotech) were diluted to a concentration of 90 nM and used to assess amino acid uptake. After incubation with ^{3}H-amino acid in the appropriate Ringer's solution, oocytes were immediately washed four times with the same ice-cold solution, individually solubilized in 1% SDS, and counted by liquid scintillation. Initial time course experiments of L-[^{3}H]leucine transport indicated that uptake was linear from 1–10 min (data not shown); consequently, time points of 2 or 5 min were chosen. For ion dependence experiments, oocytes were initially rinsed three times in Na^+ or Cl^--free solution.

RESULTS AND DISCUSSION

hATB^{0+} Cloning Strategy—Sequence homology is commonly used to identify novel genes of emerging gene families. The expressed sequence tag (EST) database was queried to identify new members of the Na^+/Cl^--dependent transporter family. After the identification of an EST (GenBank accession number AA526963) from mammary gland cDNA with homology to this family, RACE of human mammary gland Marathon Ready™ cDNA was performed to clone the full-length gene. hATB^{0+} cDNA is 4.5 kb in length and has been submitted to GenBank (GenBank accession number AF151978). hATB^{0+} possesses absolute identity with human ESTs (GenBank accession numbers AA526963, AA552658, and AA541466) and is highly similar (~90%) to mouse ESTs (GenBank accession numbers AI006618, AI006510, AA592728, AI1429024, and AI605513).

Primary Structure—hATB^{0+} is a member of the Na^+/Cl^--dependent neurotransmitter transporter family and shows the highest similarity (~60%) to the glycine transporters GLYT1 (40–42) and GLYT2 (43) and the proline transporter PROT (44). The isolated hATB^{0+} cDNA contains an open reading frame of 1926 base pairs, which predicts a protein of 642 amino acids (Fig. 1*A*). Hydrophobicity prediction (37, 45) of the primary amino acid sequence suggests 12 putative membrane-spanning domains, similar to other Na^+/Cl^--dependent transporters (Fig. 1*A*). Analysis of the amino acid sequence by ScanProsite (36) reveals several consensus sites for post-translational modification (Fig. 1*A*). There are seven possible glycosylation sites on the second putative extracellular loop and one on the third putative extracellular loop. Two consensus sites for protein kinase C phosphorylation are located at Ser-40 and Ser-261. The Ser-40 site is also present in the amino acid transporters hGLYT2 and hPROT, and the protein kinase C consensus site located at Ser-261 is highly conserved among the Na^+/Cl^--dependent neurotransmitter transporter family (20). Phorbol esters, protein kinase C activators, have been reported to regulate Na^+/Cl^--dependent transporter activity (46–50) and membrane localization (51, 52). However, regulation may or may not be mediated by direct phosphorylation by protein kinase C (47, 53). hATB^{0+} also shows a consensus site for phosphorylation by casein kinase II in the fourth putative intracellular loop at Thr-434.

Genomic Structure—Periodic query of GenBank revealed genomic sequences that were identical to hATB^{0+} (GenBank accession number AL034411 and Z96810), and alignment with hATB^{0+} cDNA predicts the gene structure. The coding sequence possesses 14 exons, each of which is ~100–200 base pairs in length (Fig. 1*B*). Genomic organization is conserved among members of the Na^+/Cl^--dependent transporter family, and the coding sequence of the transmembrane domains is not interrupted by introns (54). The genomic sequences (GenBank accession numbers AL034411 and Z96810) were assigned to

A

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MDKLKCPSFFKCREKEKVSASSENFHVGENDENQDRGNWSKKSDYLLSMI   50
GYAVGLGNVWRFPYLTYSNGGGAFLIPYAIMLALAGLPLFFLECSLGQFA  100
SLGPVSVWRILPLFQGVGITMVLISIFVTIYYNVIIAYSLYYMFASFQSE  150
LPWKNCSSWSDKNCSRSPIVTHCNVSTVNKGIQEIIQMNKSWVDINNFTC  200
INGSEIYQPGQLPSEQYWNKVALQRSSGMNETGVIVWYLALCLLLAWLIV  250
GAALFKGIKSSGKVVYFTALFPYVVLLILLVRGATLEGASKGISYYIGAQ  300
SNFTKLKEAEVWKDAATQIFYSLSVAWGGLVALSSYNKFKNNCFSDAIVV  350
CLTNCLTSVFAGFAIFSILGHMAHISGKEVSQVVKSGFDLAFIAYPEALA  400
QLPGGPFWSILFFFMLLTLGLDSQFASIETITTTIQDLFPKVMKKMRVPI  450
TLGCCLVLFLLGLVCVTQAGIYWVHLIDHFCAGWGILIAAILELVGIIWI  500
YGGNRFIEDTEMMIGAKRWIFWLWWRACWFVITPILLIAIFIWSLVQFHR  550
PNYGAIPYPDWGVALGWCMIVFCIIWIPIMAIIKIIQAKGNIFQRLISCC  600
RPASNWGPYLEQHRGERYKDMVDPKKEADHEIPTVSGSRKPE          642
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B

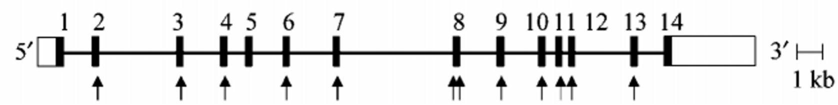

FIG. 1. **hATB^{0+} primary structure and genomic organization.** *A*, hATB^{0+} cDNA encodes a protein of 642 amino acids and possesses 12 putative transmembrane domains (*underlined*) determined by the TMpred program (37) and alignment with other Na^+/Cl^--dependent neurotransmitter transporters. hATB^{0+} contains eight consensus sites for *N*-glycosylation represented by ■ at amino acids 155, 163, 174, 189, 197, 202, and 230 in the second putative extracellular loop and at amino acid 302 in the third putative extracellular loop. Potential protein kinase C consensus sites (Ser-40 and Ser-261) are indicated by ●. One casein kinase II consensus site (Thr-434) is depicted by ○. *B*, alignment of the cDNA sequence with the genomic sequences (GenBank accession numbers AL034411 and Z96810) from chromosome X, regions Xq24 and Xq22.1–23, respectively, predicts 14 exons of 100–200 base pairs. Coding regions, ■; untranslated regions, □. *Arrows* indicate putative transmembrane domains.

chromosome X at positions Xq24 and Xq22.1–23, respectively. Interestingly, several forms of nonspecific mental retardation and other central nervous system disorders have been mapped to this region (55–62).

Tissue Distribution—A human Master blot was probed to determine the tissue distribution of hATB^{0+} mRNA. The highest expression was detected in the lung, fetal lung, trachea, and salivary gland, and lower levels of expression were detected in the mammary gland, stomach, and pituitary gland. Hybridization in the colon, uterus, prostate, and testis was very low (Fig. 2*A*). ESTs from human mammary gland, colon, and prostate and from mouse colon are in agreement with the Master blot tissue distribution data. A multiple tissue Northern blot showed no expression in the heart, brain, placenta, liver, skeletal muscle, kidney; or pancreas; however, transcripts of ~4.5 and ~2 kb were detected in the lung (Fig. 2*B*). The predominant transcript of ~4.5 kb corresponds to the length of the hATB^{0+} isolated cDNA, 4.5 kb.

Functional Studies—The application of substrate generated ionic current for all members of the Na^+/Cl^--dependent transporter family studied electrophysiologically (63). We therefore utilized a two-electrode voltage clamp to functionally characterize hATB^{0+} in the *Xenopus* oocyte expression system. Oocytes injected with hATB^{0+} cRNA generated inward current in response to the application of neutral and cationic amino acids. The negatively charged amino acids, glutamate and aspartate, evoked no current. We never observed a current greater than 2 nA in uninjected or water-injected oocytes in response to the application of 1 mM of each amino acid. Despite some seasonal and batch-to-batch variation in expression levels, more than 20 batches of oocytes injected with hATB^{0+} cRNA responded to neutral and cationic amino acids. Amino acid-induced inward current was observed at all voltages from −140 to +40 mV, and the current was increased at more negative potentials (data not shown). Fig. 3*A* illustrates the typical current evoked by increasing concentrations of amino acid (1 μM to 1 mM of phenylalanine). Dose-response data for all amino acids that generated current were saturable. Data were initially fit to the Hill equation, and Hill coefficients were determined to be approximately 1. Subsequently, data were fit to a curve assuming a Hill coefficient equal to 1. Table I presents the EC_{50} values for all amino acids evoking transport current. hATB^{0+} preferred hydrophobic amino acids but also had significant affinity for other neutral and cationic amino acids. The apparent affinity for nonpolar amino acids seems to increase with R group size, and the apparent affinity for polar amino acids seems to decrease with R group size. The affinity for proline was very low (EC_{50} > 5 mM) and probably would not be physiologically relevant.

In addition to the 20 naturally occurring amino acids, related compounds with modified side chain or "core" amino acid structure were tested. D-Tyrosine evoked transport current with an EC_{50} > 1 mM compared with L-tyrosine with an EC_{50} of 92 μM, indicating that hATB^{0+} recognizes amino acids stereospecifically. In addition, β-alanine and 3,4-dihydroxyphenylalanine (but not γ-aminobutyric acid, choline, taurine, and thyroxine) evoked inward current at concentrations of 1 mM (data not shown). hATB^{0+} had broad substrate specificity compared with its most similar family members, GLYT1 and GLYT2, which only transport glycine and glycine derivatives (40, 43), and PROT, which transports proline with the highest affinity but also transports phenylalanine, histidine, and cysteine (44). Interestingly, an insect K^+-coupled amino acid transporter, KAAT1, with sequence similarity to this family was also found to transport a broad range of amino acids (64).

The current generated by amino acid application is believed to reflect transport across the plasma membrane, but in order to verify the physical translocation of the amino acid into the cell, ^{3}H-amino acid uptake experiments were conducted (Fig. 3, *B–D*). Consistent with electrophysiological data, oocytes injected with hATB^{0+} cRNA showed higher uptake rates for L-[^{3}H]leucine and L-[^{3}H]arginine but not for L-[^{3}H]glutamate when compared with uninjected oocytes (Fig. 3, *B* and *C*). The difference in uptake rate between leucine and arginine (Fig. 3, *B* and *C*) is in agreement with the differences in EC_{50} values between leucine- and arginine-induced transport current (Table I). L-[^{3}H]Leucine uptake was significantly attenuated in the presence of 1 mM leucine (data not shown), 1 mM L-arginine, and 1 mM L-glutamine, but not in the presence of 1 mM L-glutamate (Fig. 3*B*). L-[^{3}H]Arginine transport was inhibited by 1 mM L-leucine (Fig. 3*C*). Because arginine inhibited uptake of

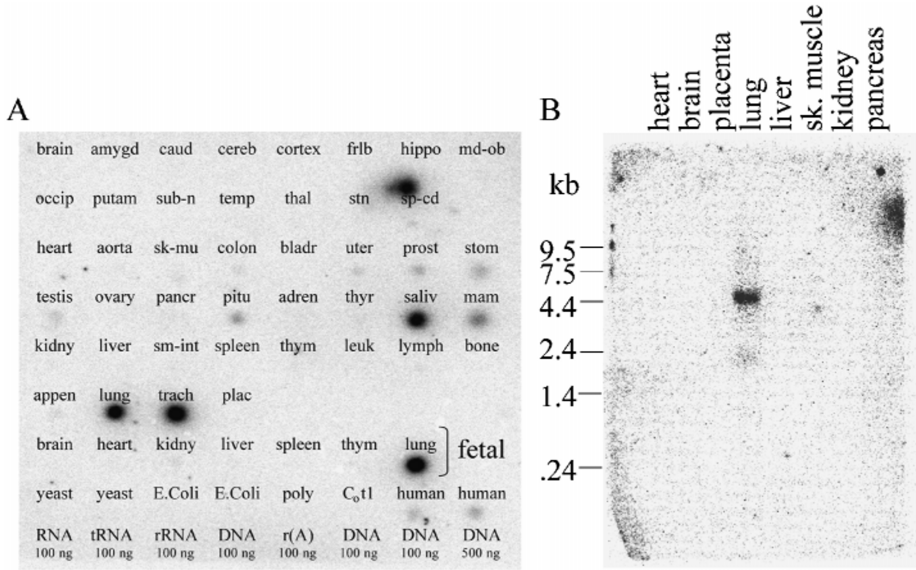

FIG. 2. **Tissue distribution of hATB^{0+} mRNA.** *A*, hybridization of a ^{32}P-labeled cDNA probe to a human Master blot (CLONTECH) was used to determine the tissue distribution of hATB^{0+} mRNA. *amygd*, amygdala; *caud*, caudate nucleus; *cereb*, cerebellum; *frlb*, frontal lobe; *hippo*, hippocampus; *md-ob*, medulla oblongata; *occip*, occipital lobe; *putam*, putamen; *sub-n*, substantia nigra; *temp*, temporal lobe; *thal*, thalamus; *stn*, subthalamic nuclei; *sp-cd*, spinal cord; *sk-mu*, skeletal muscle; *bladr*, bladder; *uter*, uterus; *prost*, prostate; *stom*, stomach; *pancr*, pancreas; *pitu*, pituitary; *adren*, adrenal gland; *thyr*, thyroid gland; *saliv*, salivary gland; *mam*, mammary gland; *kidny*, kidney; *sm-int*, small intestine; *thym*, thymus; *leuk*, peripheral leukocyte; *bone*, bone marrow; *appen*, appendix; *trach*, trachea; *plac*, placenta. Bottom row samples are negative controls: yeast total RNA, yeast tRNA, *E. coli* rRNA, *E. coli* DNA, Poly r(A), and human C_ot1 DNA. Positive controls include 100 and 500 ng of human DNA. The highest level of expression was detected in the lung, fetal lung, trachea, and salivary gland, and lower levels of expression were detected in the mammary gland, stomach, and pituitary gland. Hybridization in the colon, uterus, prostate, and testis was weak but detectable. *B*, hybridization with the same probe to a Multiple Tissue Northern blot (CLONTECH) revealed two transcripts of ~4.5 and ~2 kb in the lung.

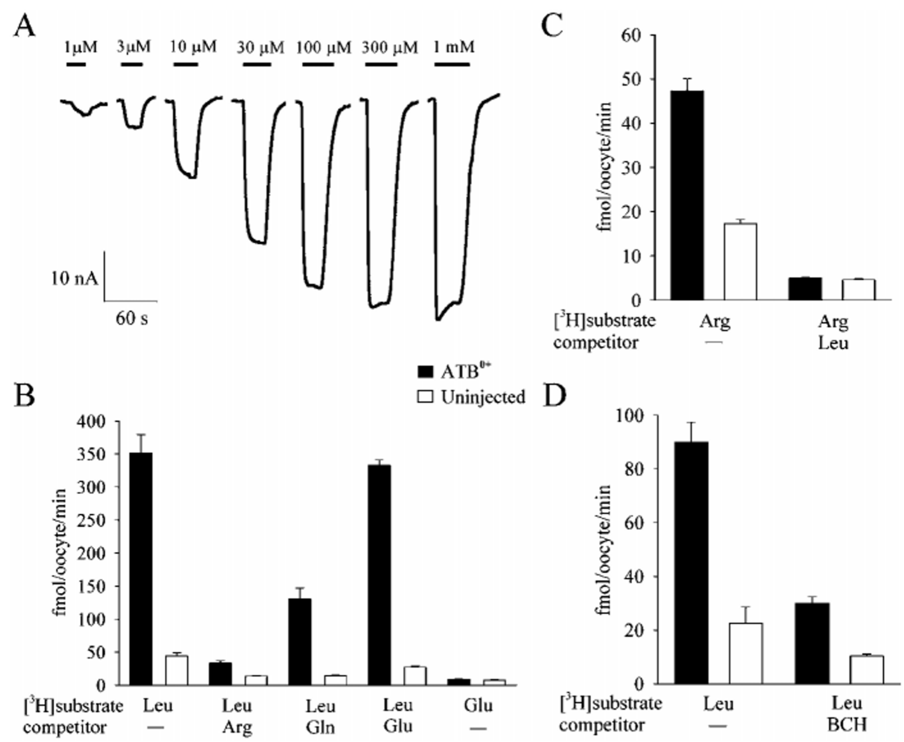

FIG. 3. **Amino acid specificity of hATB^{0+}.** *A*, a *Xenopus* oocyte expressing hATB^{0+} was voltage clamped at −80 mV. Superfusion of increasing concentrations of phenylalanine (1 µM to 1 mM, as indicated) generated increasing inward current. For *B* and *C*, hATB^{0+}-injected oocytes are represented by ■, and uninjected oocytes are represented by □. *B*, oocytes were incubated for 2 min in the presence of 90 nM L-[^{3}H]leucine or L-[^{3}H]glutamate in Ringer's solution alone or Ringer's solution containing 1 mM competing amino acid. Injection of hATB^{0+} cRNA increased the uptake of L-[^{3}H]leucine but not L-[^{3}H] glutamate compared with uninjected cells. hATB^{0+} transport of L-[^{3}H]leucine is inhibited by L-arginine and L-glutamine but not L-glutamate. *Bars* represent the mean of 10 oocytes ± S.E. *C*, oocytes were incubated for 2 min in the presence of 90 nM L-[^{3}H]arginine in Ringer's solution alone or Ringer's solution supplemented with 1 mM L-leucine. L-[^{3}H]Arginine uptake was inhibited in the presence of 1 mM L-leucine. *Bars* represent the mean of 10 oocytes ± S.E. *D*, oocytes were incubated for 5 min in the presence of 90 nM L-[^{3}H] leucine in Ringer's solution or Ringer's solution supplemented with 10 mM BCH. BCH significantly inhibits L-[^{3}H]leucine uptake. *Bars* represent the mean of 10 oocytes ± S.E.

L-[^{3}H]leucine, and leucine inhibited uptake of L-[^{3}H]arginine, we conclude that both of these amino acids are carried by the same transport system. The difference in L-[^{3}H]leucine inhibition by arginine and glutamine (90% and 63%, respectively) is probably a result of the difference in their apparent affinity for hATB^{0+} (Table I). Uptake experiments demonstrated that hATB^{0+} transports neutral and cationic amino acids (*e.g.* arginine and leucine). The combination of electrophysiology and uptake experiments indicates that the current measured represents the transport process. The transport current measurements can therefore be used to assess hATB^{0+} substrate specificity and affinity for all amino acids tested.

Pharmacological studies are an important tool for amino acid transport system classification. High concentrations (5–10 mM) of 2-aminobicyclo-[2.2.1]-heptane-2-carboxylic acid (BCH), a cyclic amino acid, have been shown to inhibit system B^{0+} amino acid transport (21–24, 26). In Fig. 3*D*, 10 mM BCH significantly inhibited hATB^{0+}-mediated L-[^{3}H]leucine uptake

TABLE I
Concentration-dependent amino acid-induced transport current

Oocytes expressing $hATB^{0+}$ were voltage clamped at −80 mV and subjected to increasing concentrations of amino acid ranging from 1 μM to 10 mM. (See representative experiment in Fig. 3*A*). Data from individual oocytes were fit to the Michaelis-Menten equation, and EC_{50} values are presented (mean ± S.E.; n = 3 or 4).

Amino acid	EC_{50}
	μM
Nonpolar	
Isoleucine	6 ± 1
Leucine	12 ± 2
Methionine	14 ± 1
Valine	36 ± 2
Alanine	99 ± 36
Glycine	111 ± 30
Proline	>5 mM
Polar	
Serine	43 ± 5
Cysteine	118 ± 33
Asparagine	348 ± 84
Threonine	405 ± 80
Glutamine	633 ± 62
Aromatic	
Phenylalanine	17 ± 1
Tryptophan	26 ± 6
Tyrosine	92 ± 10
Charged	
Histidine	76 ± 20
Lysine	100 ± 1
Arginine	104 ± 35
Aspartate	N/D[a]
Glutamate	N/D

[a] N/D, not detectable.

by 67%. BCH was also evaluated electrophysiologically; the application of 10 mM BCH resulted in an inward current of 5.8 ± 1.8 nA (mean ± S.E.; n = 3). Because BCH generates current and inhibits L-[^{3}H]leucine uptake, it is probably a competitive substrate for $hATB^{0+}$.

Members of the Na^+/Cl^--dependent transporter family require both Na^+ and Cl^- for transport to occur (65). Fig. 4*A* illustrates that $hATB^{0+}$ L-[^{3}H]leucine transport was strongly dependent on Na^+ and Cl^- ions. The $hATB^{0+}$-related component of L-[^{3}H]leucine uptake was found to decrease by >99% in Na^+-free and Cl^--free solutions. In agreement with uptake data, no transport current was measured in Na^+-free solution (Fig. 4*B, inset*). On the other hand, a small but significant current was generated by 100 μM leucine in Cl^--free solution. This current was approximately 6% of the total current evoked in the presence of 108 mM Cl^- (Fig. 4*C, inset*). A similar small Cl^--independent current was also reported for the γ-aminobutyric acid transporter, GAT1, expressed in *Xenopus* oocytes (39), indicating that external Cl^- is not absolutely required for some transport to occur. Fig. 4, *B* and *C,* describes the effect of increasing concentrations of Na^+ or Cl^- on L-leucine (100 μM)-induced transport current. Dose-response curves were fitted to the Hill equation. For Na^+, a Hill coefficient of 2.3 ± 0.13, an EC_{50} of 7.4 ± 0.24 mM, and an I_{max} of 31 ± 1.6 nA were determined (mean ± S.E.; n = 5). The Cl^- data yielded a Hill coefficient of 0.92 ± 0.07, an EC_{50} of 0.61 ± 0.03 mM, and an I_{max} of 32 ± 2.5 nA (mean ± S.E.; n = 5). EC_{50} values of 7.4 and 0.61 mM for Na^+ and Cl^-, respectively, indicate that under physiological conditions, these ions are not rate-limiting for amino acid transport. A Hill slope of >2 for Na^+ suggests that the transport cycle involves the binding of at least two Na^+ ions, and Hill slopes for Cl^- and amino acids close to 1 suggest the binding of one Cl^- ion and one amino acid. Therefore, we propose a transport stoichiometry of 2 or 3 Na^+, 1 Cl^-, and 1 amino acid.

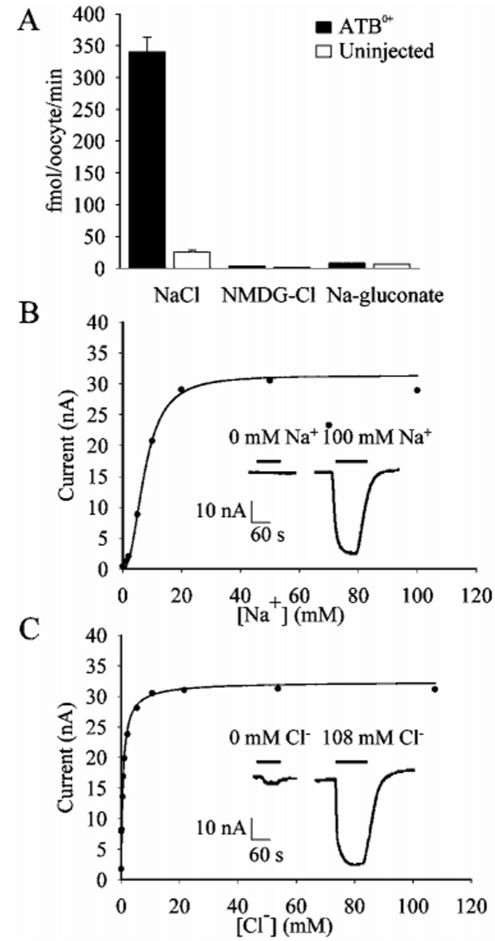

FIG. 4. **Amino acid transport is Na^+- and Cl^--dependent.** *A*, $hATB^{0+}$-injected oocytes (■) and uninjected oocytes (□) were incubated in the presence of 90 nM L-[^{3}H]leucine in Ringer's solution, Na^+-free solution (*N*-methyl-D-glucamine substitution), or Cl^--free solution (gluconate substitution). Na^+ and Cl^- substitution virtually eliminated $hATB^{0+}$-mediated L-[^{3}H]leucine uptake (~99%). *B* and *C*, $hATB^{0+}$-injected oocytes were voltage clamped at −80 mV. Current was recorded during the application of 100 μM L-leucine in the presence of increasing concentrations of Na^+ (*B*) or Cl^- (*C*). *Inset*, an oocyte voltage clamped at −80 mV in response to 100 μM L-leucine in Ringer's solution, Na^+-free solution, or Cl^--free solution. Data from individual oocytes were fit to the Hill equation. *B*, for Na^+ dose-response experiments, a Hill coefficient of 2.3 ± 0.13, an EC_{50} of 7.4 ± 0.24 mM, and an I_{max} of 31 ± 1.6 were determined (mean ± S.E.; n = 5). *C*, for Cl^- substitution experiments, the Hill coefficient, EC_{50}, and I_{max} were 0.92 ± 0.07, 0.61 ± 0.03 mM, and 32 ± 2.5 nA, respectively (mean ± S.E.; n = 5).

Possible Physiological Significance of $hATB^{0+}$—Amino acid transport through $hATB^{0+}$ can be summarized by the following characteristics: 1) inward current associated with neutral and cationic amino acid application; 2) uptake of and competitive inhibition by neutral and cationic amino acids but not anionic amino acids; 3) low affinity for proline; 4) uptake inhibition by the competitive substrate BCH (5–10 mM); and 5) Na^+ and Cl^- dependence. The properties of $hATB^{0+}$ are similar to the properties of a transport system originally described in mouse blastocysts, system B^{0+} (22, 66, 67). System $B^{0+}$is defined by Na^+-coupled transport of neutral and cationic amino acids. This transport system also shows sensitivity to BCH at high concentrations (5–10 mM) (22–24, 26). The similarity of amino acid specificity, ion dependence, and BCH sensitivity suggest that $hATB^{0+}$ is the first transporter to possess all system B^{0+} characteristics.

System B^{0+}-like transport has also been reported in *Xenopus* oocytes (23–26), a human intestinal cell line (27), rabbit small intestine (28), rabbit conjunctiva (29, 30), rat pituitary gland

(31), bullfrog lung (32), and human lung (33). Several studies have shown system B^{0+} amino acid transport in *Xenopus* oocytes (23–26), and these data are confirmed by our results. In the presence of 1 mM arginine or leucine, the endogenous uptake of L-[^{3}H]leucine (Fig. 3*B*) and L-[^{3}H]arginine (Fig. 3*C*), respectively, was attenuated. BCH inhibited L-[^{3}H]leucine uptake by uninjected cells (54%) (Fig. 3*D*). Endogenous L-[^{3}H]leucine uptake was also Na^+- and Cl^--dependent (Fig. 4*A*). These data, specifically the Cl^- dependence of L-[^{3}H]leucine uptake, suggest the expression of a hATB^{0+}-like transporter in *Xenopus* oocytes.

The pituitary gland is of special interest because amino acids (*e.g.* arginine and leucine) are known to act as secretagogues for anterior pituitary hormones (68). Amino acid-induced hormone secretion was found to be induced by an intracellular rise in Ca^{2+} and dependent on extracellular Na^+ (31). Based on the amino acid specificity that caused an increase in intracellular Ca^{2+}, Villalobos *et al.* (31) hypothesized that the amino acid influx is through a Na^+-dependent transporter. Similar substrate specificity and the possible expression of hATB^{0+} in the pituitary gland suggest that hATB^{0+} may play a role in amino acid-induced pituitary secretion. A transporter could regulate hormone secretion as a direct result of Na^+, Cl^-, or amino acid influx or due to depolarization of the cell membrane. These hypotheses are currently being investigated in our laboratory.

Transport measurements in lung epithelial cells provide the strongest evidence for hATB^{0+}-mediated system B^{0+} amino acid transport. Galietta *et al*. (33) reported a potential B^{0+} transport system in cultured human bronchial epithelial cells using uptake and short circuit current measurements. They observed Na^+-dependent transport current in response to the application of L-arginine, L-lysine, and L-alanine with EC_{50} values of 80, 66, and 26 μM, respectively, and a much lower affinity for proline. Also, L-aspartate and taurine did not produce short circuit current in these cells. The reported amino acid transport in human bronchial epithelial cells may be mediated by hATB^{0+} due to its high expression in the lung and corresponding substrate specificity and affinity. Therefore, hATB^{0+} could play a significant role in the removal of amino acids, Na^+, and Cl^- from the airway surface liquid. Localization of mRNA and protein and more extensive functional measurements will determine whether hATB^{0+} underlies the amino acid transport in the lung, pituitary gland, and other tissues in which system B^{0+}-like amino acid transport has been described.

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Note Added in Proof—After acceptance for publication, the mouse homolog of hATB^{0+}, was cloned. The sequence has been submitted to the GenBank™/EBI Data Bank with accession number AF161714.

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