We have cloned the human Na\(^+\)- and H\(^+\)-coupled amino acid transport system N (hSN1) from HepG2 liver cells and investigated its functional characteristics. Human SN1 protein consists of 504 amino acids and shows high homology to rat SN1 and rat brain glutamine transporter (GlnT). When expressed in mammalian cells, the transport function of human SN1 could be demonstrated with glutamine as the substrate in the presence of LiCl (instead of NaCl) and cysteine. The transport activity was saturable, pH-sensitive, and specific for glutamine, histidine, asparagine, and alanine. Analysis of Li\(^+\) activation kinetics showed a Li\(^+\):glutamine stoichiometry of 2:1. When expressed in *Xenopus laevis* oocytes, the transport of glutamine or asparagine via human SN1 was associated with inward currents under voltage-clamped conditions. The transport function, monitored as glutamine- or asparagine-induced currents, was saturable, Na\(^+\)-dependent, Li\(^+\)-tolerant, and pH-sensitive. The transport cycle was associated with the involvement of more than one Na\(^+\) ion. Uptake of asparagine was directly demonstrable in these oocytes by using radiolabeled substrate, and this uptake was inhibited by membrane depolarization. In addition, simultaneous measurement of asparagine influx and charge influx in the same oocyte yielded an asparagine:charge ratio of 1. These data suggest that SN1 mediates the influx of two Na\(^+\) and one amino acid substrate per transport cycle coupled to the efflux of one H\(^+\), rendering the transport process electrogenic.

Glutamine, the most abundant amino acid in blood, is involved in several metabolic pathways. It plays an important role in ammonia metabolism (1), in the synthesis of purines and pyrimidines (2), and in the glutamine-glutamate cycle that occurs between neurons and glial cells in the brain (3, 4) and between placenta and liver in the developing fetus (5). Glutamine is also an obligatory participant in the intercellular glutamine cycle that occurs in the liver between periportal hepatocytes and perivenous hepatocytes (6). Cellular uptake of glutamine is known to be mediated by at least three different Na\(^+\)-dependent amino acid transport systems, namely system A, system B\(^0\), and system N (7–9). The first two transport systems have a broad substrate specificity accepting several neutral amino acids, including glutamine as substrates. In contrast, system N exhibits a much narrower substrate specificity (10). It transports only glutamine and asparagine, and in some instances, histidine. The tissue distribution pattern is significantly different for these three transport systems. System A is expressed ubiquitously in mammalian tissues, whereas system B\(^0\) has been described primarily in the intestine and kidney and system N in the liver, skeletal muscle, and brain (7–9). Functional studies have indicated that system N expressed in the liver, skeletal muscle, and brain may not be identical (10–12). The differences in the characteristics of system N in these three tissues have led to the following subtype classification of this transport system: system N in the liver, system N\(^m\) in the skeletal muscle, and system N\(^o\) in neurons. All three subtypes transport glutamine and asparagine in a Na\(^+\)-dependent manner. Differences lie primarily in Li\(^+\) tolerance and pH sensitivity. The liver system N functions well even when Na\(^+\) is replaced by Li\(^+\). Thus, this subtype is Li\(^+\)-tolerant. In addition, it is highly pH-sensitive and its transport activity is almost undetectable at pH 7 or below (10). On the contrary, the subtypes N\(^m\) and N\(^o\) are comparatively Li\(^+\)-tolerant and pH-insensitive (11, 12). The subtype N\(^o\) can, however, be differentiated from the other two subtypes by its ability to interact with glutamate (10, 12).

Recent cloning studies have begun to unravel the molecular nature of these transport systems. We have cloned system B\(^0\) (ATB\(^0\) or ASCT2) and characterized its ability to transport glutamine and other neutral amino acids in a Na\(^+\)-coupled manner (13–15). On the basis of the primary structure, ATB\(^0\) belongs to the family of glutamate transporters (16). Moreover, Varoqui *et al.* (17) reported on the cloning of a Na\(^+\)-coupled transporter for glutamine and the system A-specific substrate \(\alpha\)-(methylamino)isobutyric acid. Surprisingly, this transporter, designated GlnT\(^1\), is expressed in neurons but not in any other tissue. This suggests that system A may also consist of distinct subtypes. GlnT represents one of the system A subtypes. We have recently cloned and characterized the ubiquitously expressed system A subtype, designated AT2A for amino acid transporter A2, the brain-specific system A subtype (GlnT) being AT1A (18). The cloning of another Na\(^+\)-coupled glutamine transporter from rat brain has been reported recently (19). This transporter is Li\(^+\)-tolerant and is expressed.

\(\text{ATB}^{0}\) and \(\text{ATB}^{1}\) are abbreviations used for GlnT, glutamine transporter; SN, system N; hSN1, human SN1; rSN1, rat SN1; GlnT, glutamine transporter; HRPE, human retinal pigment epithelial; kbp, kilobase pairs; RT-PCR, reverse transcription-polymerase chain reaction; Mes, 4-morpholineethanesulfonic acid; bp, base pair(s).

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primarily in the brain and liver. Based on functional characteristics and tissue distribution pattern, this transporter has been identified as system N and designated SN1 (19). Functional analysis of rat SN1 has led to the discovery of a unique feature of this transporter. SN1 mediates the influx of Na\(^+\) and glutamine into the cells in exchange with intracellular H\(^+\). This is indicated by the glutamine-dependent intracellular alkalinization in SN1-expressing cells. Thus, SN1 is a Na\(^+\)- and H\(^+\)-coupled glutamine transporter. These findings agree with the pH sensitivity of system N observed in hepatocytes (10). There is no information available for system N in the liver on the role of membrane potential in the transport process. However, functional studies with cloned rat SN1 have led to the conclusion that the transport process is electroneutral, suggesting a Na\(^+\)-glutamate-H\(^+\) stoichiometry of 1:1:1. On the basis of primary structure, SN1, ATA1, and ATA2 are related to each other. Interestingly, these three plasma membrane transport proteins bear significant homology to the vesicular \(\gamma\)-aminobutyrate transporter, which is present in synaptic vesicles in the brain (20). This synaptic vesicle transporter mediates the entry of \(\gamma\)-aminobutyrate into the vesicles in exchange for intravesicular H\(^+\) (20, 21), a functional feature similar to that of rat SN1. ATA1 and ATA2 are also highly pH-sensitive, but whether or not H\(^+\) is a transportable substrate for these two transporters is not known.

In this report we describe the cloning of the human SN1 from the HepG2 liver cell line and the structural organization of the sn1 gene. We also demonstrate here the transport function of human SN1 using two different heterologous expression systems with mammalian cells and Xenopus laevis oocytes. Human SN1 transports glutamine in a Na\(^+\)-dependent manner. It is also H\(^+\)-coupled and Li\(^+\)-tolerant. Most importantly, the present studies show that the transport process mediated by human SN1 is electronegic with a Na\(^+\)-glutamate stoichiometry of 2:1, contrary to the conclusions drawn by Chaudhry et al. (19) with rat SN1. To determine whether this difference is due to species-dependent variation in the transport mechanism, we cloned rat SN1 from skeletal muscle and studied its transport function. Our results show that rat SN1 is also electronegic similar to human SN1. These data suggest that the transport mechanism of SN1 involves the influx of two Na\(^+\) ions and one glutamine molecule coupled to the efflux of one H\(^+\).

**EXPERIMENTAL PROCEDURES**

**Materials**—[3H]Glutamine (specific radioactivity, 49.9 Ci/mmol) was purchased from NEN Life Science Products. [3H]Asparagine (specific radioactivity, 650 mCi/mmol) was purchased from Moravek (Brea, CA). Human retinal pigment epithelial (HRPE) cells were originally provided by Dr. M. A. Del Monte (University of Michigan, Ann Arbor, MI) and have been in use in our laboratory for several years for heterologous expression of a variety of cloned transporters (22–24). The human liver cell line HepG2 was obtained from the American Type Culture Collection (Manassas, VA). Cell culture media, TRIzol reagent, oligo(dT)-cellulose, and Lipofectin were from Life Technologies (Gaithersburg, MD). Restriction enzymes were either from Promega or from New England BioLabs. Magna nylon transfer membranes used in the library screening were from Micron Separations, Inc. (Westboro, MA). The Ready-to-Go oligolabeling kit was purchased from Amersham Pharmacia Biotech.

**Probe Preparation**—The recently cloned rat SN1 is highly homologous to the human cDNA designated g17 in the GenBank data base (accession number U49082). This indicated that g17 most likely represents the human homolog of rat SN1. Therefore, to clone the full-length g17 cDNA for functional studies, we prepared a cDNA fragment by reverse transcription-polymerase chain reaction (RT-PCR) using primers based on the nucleotide sequence of g17. The sense primer was 5\'-AACATCGGAGCAGCTTGCCG3\'-, which corresponded to nucleotide positions 581–600 in g17 cDNA sequence, and the antisense primer was 5\'-AAGGTAGGTAGCGAGAG3\'-, which corresponded to nucleotide positions 1136–1155 in g17 cDNA sequence. Because the Northern blot analysis has shown that rat SN1 mRNA is expressed most abundantly in the liver (19), we used poly(A)\(^+\) mRNA isolated from HepG2 cells, a human liver cell line, as a template for RT-PCR. A single product of expected size (approximately 0.6 kilobase pair) was obtained in the RT-PCR reaction. This product was subcloned into pGEM-T vector and sequenced to establish its molecular identity. This cDNA fragment was used as a probe to screen a cDNA library.

**Construction of cDNA Libraries**—The SuperScript plasmid system (Life Technologies, Inc.) was used to establish unidirectional cDNA libraries with poly(A)\(^+\) mRNA isolated from HepG2 cells and rat skeletal muscle. Poly(A)\(^+\) mRNA was prepared by subjecting total RNA twice to oligo(dT)-cellulose affinity chromatography prior to use in library construction. The cDNA products with sizes greater than 1 kb were separated by size-fractionation and used for ligation at a SalI/NotI site in spsSORT1 vector.

**cDNA Library Screening**—The 0.6-kbp cDNA fragment of g17 was labeled with [\(\alpha\)-32P]dCTP using the Ready-to-Go oligolabeling kit. The HepG2 and rat skeletal muscle cDNA libraries were screened with this probe under low stringency conditions. Hybridization was carried out for 20 h at 60 °C in a solution containing 5 × SSPE (1 × SSPE = 0.15 M Na\(\text{H}_{2}\text{PO}_4\), 1.8 M CaCl\(_2\), 0.8 M MgSO\(_4\), 10 mM cysteine, and 5 mM glucose) at pH 8.5. The hybridization washing was done as described earlier (22–24), which involved extensive washes with 3 × SSPE, 0.5% SDS at room temperature. Positive clones were identified, and the colonies were purified by secondary screening.

**DNA Sequencing**—Both sense and antisense strands of the cDNAs were sequenced by primer walking. Sequencing by the dideoxynucleotide chain termination method was performed by Toy DyeDeoxy terminator cycle sequencing with an automated PE Biosystems 377 Prism DNA sequencer. The sequence was analyzed using the GCG sequence analysis software package GCG, version 7.8 (Genetics Computer Group, Inc. Madison, WI).

**Functional Expression in HRPE Cells**—This was done using the vaccinia virus expression system as described below (22–24). Subconfluent HRPE cells grown on 24-well plates were first infected with a recombinant vaccinia virus (VT1–3) encoding T7 RNA polymerase and then transfected with the plasmid carrying the full-length cDNA. After 10–12 h post-transfection, uptake measurements were made at 37 °C with 50 μM [3H]glutamine. In most experiments, the uptake medium was 25 mM Tris/Hepes, pH 8.5, containing 140 mM LiCl, 5.4 mM KCl, 1.8 mM CaCl\(_2\), 0.8 mM MgSO\(_4\), 10 mM cysteine, and 5 mM glucose. The incubation was 15 min. Endogenous transport was always determined in parallel using cells transfected with empty vector. This transport accounted for 15–25% of the transport measured in cells that were transfected with the vector carrying the cDNA insert. Therefore, the transport values measured in cells transfected with empty vector were always subtracted from the corresponding transport values measured in cells transfected with vector-cDNA to obtain the cDNA-specific uptake. The cDNA-induced increase in the maximal uptake was always determined at pH 8.5 in a LiCl-containing uptake medium (instead of NaCl) and in the presence of 10 mM cysteine. The influence of pH on the transport function was measured by the uptake of 50 μM [3H]glutamine in cDNA-transfected cells and in vector-transfected cells at varying pH at varying concentrations of LiCl and at varying concentrations of glutamine (0.1–15 mM). The kinetic parameters, Michaelis-Menten constant (K\(_M\)) and maximal velocity (V\(_\text{max}\)), were calculated by fitting the data to a Michaelis-Menton equation describing a single saturable transport system. Analysis was done by nonlinear regression, and the resultant values for the kinetic parameters were confirmed by linear regression. Li\(^+\) activation kinetics were analyzed by measuring cDNA-specific transport of glutamine at varying concentrations of Li\(^+\) (10–80 mM). The osmolality of the buffer and the concentration of Cl\(^-\) were kept constant by substituting LiCl with N-methyl-D-glutamine hydroxide. The data fit to the Hill equation in a single saturable transport system. The Hill coefficient was calculated by nonlinear regression as well as by linear regression.

**Functional Expression in X. laevis Oocytes**—cRNA from the cloned cDNA was synthesized using the MegAscript kit (Ambion) according to the manufacturer’s protocol. The cDNA was linearized using NotI, and the cDNA insert was transcribed in vitro using T7 RNA polymerase in the presence of a RNA cap analog. The resultant cRNA was purified by...
multiple extractions with phenol/chloroform and precipitated with ethanol.

Mature oocytes from *X. laevis* were isolated by treatment with collagenase A (1.6 mg/ml), manually defolliculated, and maintained at 18 °C in modified Barth's medium supplemented with 10 mg/ml gentamicin, 100 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 3 mM HEPES, 3 mM Mes, and 3 mM Tris, pH 8.0. Oocytes were used for electrophysiological studies 6 days after RNA injection. Electrophysiological studies were done by the conventional two-microelectrode voltage clamp method (26–28). Oocytes were superfused with a NaCl-containing buffer (100 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 3 mM HEPES, 3 mM Mes, and 3 mM Tris, pH 8.0) followed by the same buffer containing different amino acid substrates. The membrane potential was held steady at −50 mV. For studies involving the current-voltage (I-V) relationship, step changes in membrane potential were applied, each for a duration of 100 ms in 20-millivolt (mV) increments. Kinetic parameters for the saturable transport of glutamine and asparagine were calculated using the Michaelis-Menten equation. Data were analyzed by nonlinear regression and confirmed by linear regression.

When the effects of Na+ on the transport (i.e. amino acid-induced currents) were evaluated, the oocyte was perfused with buffer containing different concentrations of Na+ and 10 mM glutamine or asparagine. The data for the Na+ -dependent activation of amino acid-induced currents were fitted to the Hill equation, and the Hill coefficient was calculated by nonlinear regression as well as by linear regression. In some experiments, the perfusion buffer contained LiCl instead of NaCl to determine if Na+ was replaceable with Li+ to support the amino acid-induced currents. When the influence of Cl− on the amino acid-induced currents was assessed, a Cl−-free buffer was used that contained gluconate salts instead of chloride salts. In experiments dealing with the influence of pH on the amino acid-induced currents, NaCl- or LiCl-containing buffers of varying pH were prepared by appropriately adjusting the concentrations of Mes, Heps, and Tris.

Uptake of [3H]asparagine in control oocytes and in human SN1-expressing oocytes was measured at pH 8.0 in the presence of NaCl as described previously (29). The concentration of [3H]asparagine (unlabeled plus radiolabeled) was 250 nM. To assess the role of membrane potential in the uptake of asparagine, uptake measurements were made in the presence of 50 mM Na+ but with low (2 mM) or high (52 mM) K+. The oocyte membrane was depolarized with the high concentration of K+. This method has been used previously in our laboratory to study the role of membrane potential in the transport function of the organic cation transporter OCT3 (30) and the Na+-dependent multivitamin transporter (31).

To assess whether there is a direct relationship between the amino acid influx and the inward currents observed in oocytes expressing human SN1, we measured simultaneously in the same oocyte the influx of asparagine and the membrane currents associated with the asparagine influx. Oocytes, clamped at −50 mV, were perfused with a NaCl-containing buffer, and current traces were monitored in a chart recorder until they reached a steady baseline. The oocyte was then perfused with 2.5 mM asparagine (unlabeled plus radiolabeled) in the same buffer for 8–10 min while the asparagine-induced currents were recorded. At the end of this period, the oocyte was perfused with the buffer in the absence of asparagine until the currents returned to the baseline levels. The oocyte was removed, rinsed three times with ice-cold buffer, and lysed in 10% sodium dodecyl sulfate. The amount of radioactivity in the oocyte lysate was then quantitated for the calculation of asparagine influx. The total inward charge transfer was calculated by integrating the area under the current versus time curve and by using the Faraday constant. The magnitude of the amino acid influx was then compared with the magnitude of the charge transfer to determine the ratio of the amino acid influx to the charge influx. This experiment utilized the Fetchex program within the pCLAMP 6.0 software package. We have used this method previously in our laboratory to determine the substrate/charge transfer ratio for the H+-coupled peptide transporter PEPT2 (32) and Na+-coupled dicarboxylate transporter NaDC3 (33).

**RESULTS**

**Structural Features of Human SN1**—A full-length human SN1 cDNA was isolated by screening a HepG2 cDNA Library with a cDNA fragment of g17 clone as a probe. The cDNA (GenBank accession no. AF244548) is 2437 bp long with an open reading frame consisting of 1515 bp (including the termination codon). The open reading frame is flanked by a 113-bp-long 5′-untranslated region and a 809-bp-long 3′-untranslated region. The cDNA codes for a protein of 504 amino acids, with a predicted molecular mass of 56 kDa. Hydroxylation analysis of the amino acid sequence suggests the presence of 12 putative transmembrane domains in the protein. There is a single putative site for N-linked glycosylation in the extracellular loop between the transmembrane domains 7 and 8 (Asn-323). The protein also contains a single protein kinase C-dependent phosphorylation site in the cytoplasmic tail (Thr-497). At the level of amino sequence, the human SN1 exhibits 90% identity and 91% similarity with the recently cloned rat SN1 (19). ATA1 (17) and ATA2 (18) also show significant homology to human SN1. Interestingly, ATA1 and ATA2 represent the two currently known subtypes of amino acid transport system A. It appears that the amino acid transporters belonging to system N and system A form a distinct family.

**Functional Characteristics of Human SN1 Expressed Heterologously in Mammalian Cells**—In the recent report of the cloning of rat SN1, the investigators used PS120 cells for functional expression of the cloned transporter (19). These cells have an acidic intracellular pH due to the deficiency of Na+-H+ exchange activity (34). The transport function of rat SN1 could not be detected in cells such as Chinese hamster ovary cells (19). In the present study, we used HRPE cells for functional analysis of human SN1. The pH of the uptake medium was 8.5 based on the known pHe dependence of system N (10). The transport function of human SN1 was not detectable with glutamine as a substrate in the regular NaCl-containing uptake medium (Fig. 1A). In fact, the transport of glutamine was lower in cDNA-transfected cells than in control cells transfected with vector alone. Because System N is Li+-tolerant, we then measured glutamine transport in control cells and in cells expressing human SN1 in an uptake medium in which NaCl was replaced with LiCl iso-osmotically (Fig. 1B). Glutamine transport in control cells and in cDNA-transfected cells decreased considerably as a result of Li+- substitution of Na+, but the transport in cDNA-transfected cells was not very much different from the transport in control cells. Thus, the transport function of human SN1 was not detectable even in the LiCl-containing medium. Glutamine transport in mammalian cells is mediated by at least three different Na+-dependent systems, system A, system B0, and system N. The identity of the systems that are responsible for glutamine uptake in HRPE cells is not known. However, the substrate specificity of system N is very narrow compared with that of system A and system B0. Therefore, we decided to reduce the background glutamine uptake in HRPE cells by including in the uptake medium an amino acid that is a substrate for system A and system B0 but not for system N. We selected cysteine for this purpose, because it can inhibit glutamine uptake that is mediated not only by the Na+-dependent systems A and B0 but also by the Na+-independent systems asp and b(+,-) (7, 9). When cysteine (10 mM) was included in the NaCl-containing uptake medium, the transport of glutamine in control cells decreased by 95%. Interestingly, the transport function of heterologously expressed human SN1 became detectable under these uptake conditions (Fig. 1C). Glutamine transport in control cells transfected with vector alone was 0.88 ± 0.03 nmol/10⁶ cells/15 min. The transport increased significantly by −55% in cDNA-transfected cells (1.36 ± 0.02 nmol/10⁶ cells/15 min). To improve the detection of cDNA-specific transport even further, we replaced NaCl in the uptake medium with LiCl but keeping cysteine at 10 mM. This maneuver reduced the background glutamine transport in control cells further, from 0.88 ± 0.03 to 0.50 ± 0.01 nmol/10⁶ cells/15 min. Under these uptake conditions, glutamine transport in cDNA-transfected cells increased by more than 3-fold (1.70 ± 0.06 nmol/10⁶ cells/15 min) (Fig. 1D). Thus,
the transport function of the cloned human SN1 became easily detectable in HRPE cells in a Li⁺-containing uptake medium in the presence of cysteine. The magnitude of stimulation of glutamine transport in human SN1 cDNA-transfected cells compared with vector-transfected cells observed under these conditions in HRPE cells was comparable to the stimulation reported by Chaudhry et al. (19) in Na⁺-H⁰ exchanger-deficient P120 cells with rat SN1. We therefore used these specific uptake conditions (i.e., LiCl-containing medium with 10 mM cysteine) to characterize the transport function of human SN1 in HRPE cells.

Fig. 2A describes the pH dependence of glutamine transport in vector-transfected cells and in human SN1 cDNA-transfected cells. The cDNA-specific transport was almost undetectable at pH 6.0. But, the transport increased gradually to a marked extent as the pH was changed from 6.0 to 8.5. The cDNA-specific transport increased almost 5-fold between pH 6.5 and 7.5 and an additional 2.5-fold between pH 7.5 and 8.5. The transport of glutamine mediated by human SN1 was saturable in the glutamine concentration range of 0.1–15 mM (Fig. 2B). The Michaelis-Menten constant (K_m) for the transport process was 1.6 ± 0.1 mM.

We then tested the substrate specificity of human SN1 (Table I). The transport of [3H]glutamine (50 μM) mediated by human SN1 was inhibited by unlabeled glutamine, histidine, asparagine, and alanine. The acidic amino acids aspartate and glutamate, the cationic amino acid arginine, and the system A-specific substrate α-(methylamino)isobutyric acid did not have any effect on the transport function of human SN1. The neutral amino acids leucine and phenylalanine also did not compete with glutamine for transport via human SN1. These neutral amino acids leucine and phenylalanine also did not have any effect on the transport function of human SN1. The pH of the medium in all four cases was 8.5.

The kinetics of the activation of human SN1 transport function by Li⁺ was then investigated (Fig. 3B). The transport of glutamine was measured in control cells and in cDNA-transfected cells at varying concentrations of Li⁺ (10–80 mM). The influence of Li⁺ on cDNA-specific transport was analyzed. The relationship between Li⁺ concentration and human SN1-mediated glutamine transport was found to be sigmoidal. When the data were fitted to the Hill equation, a value of 2.0 ± 0.2 was obtained for the Hill coefficient (Fig. 3, inset). This suggests that two Li⁺ ions are involved per transport cycle for the activation of glutamine transport by human SN1. Therefore, the Li⁺:glutamine stoichiometry for the transport process is 2:1.

Electrophysiological Characteristics of Human SN1 Expressed Heterologously in X. laevis Oocytes—The involvement of two Li⁺ ions per transport cycle in human SN1-mediated transport process was an unexpected finding, because Chaudhry et al. (19) have recently concluded that the transport process mediated by rat SN1 is electroneutral. SN1 catalyzes the influx of Na⁺ (or Li⁺) and glutamine into the cells in exchange for intracellular H⁺. The H⁺ efflux associated with glutamine influx has been clearly demonstrated for rat SN1 by intracellular pH measurements (19). Human SN1 is also likely to mediate the efflux of H⁺ coupled to the influx of Na⁺ (or Li⁺) and glutamine based on the marked pH sensitivity of the transport process (Fig. 2A). If the SN1-mediated transport process is electroneutral, one would expect a Na⁺ (or Li⁺):glutamine stoichiometry of 1:1 with the efflux of one H⁺. The stoichiometry analysis was, however, not done for rat SN1 to corroborate the conclusion that the transport process is electroneutral. Our present findings that the Li⁺:glutamine stoichiometry is 2:1 suggest that the transport mediated by human SN1 is electrogenic with a net transfer of a positive charge into the cells. To confirm the electrogenic nature of human SN1, we expressed the transporter in X. laevis oocytes and assessed its transport.
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HRPE cells were transfected with either pSPORT vector alone or pSPORT-human SN1 cDNA. Functional expression was carried out using the vaccinia virus expression technique. Transport of 50 μM [3H]glutamine was measured in cells using a LiCl-containing medium (pH 8.5) with 10 mM cysteine. Transport assay was done at 37°C for 15 min. When present, the concentration of unlabeled amino acids was 5 mM. Values (means ± S.E.) are from two independent transfection experiments, each done in triplicate. Values in parentheses are percentage of control measured in the absence of inhibitors.

| Unlabeled amino acid | [3H]Glutamine transport |
|----------------------|-------------------------|
|                      | pSPORT                  | hSN1 cDNA               | cDNA-specific                      |
|                      | nmo1/106 cells/15 min    | nmo1/106 cells/15 min    | nmo1/106 cells/15 min              |
| Control              | 0.67 ± 0.16              | 2.54 ± 0.19              | 1.87 (100)                         |
| Histidine            | 0.45 ± 0.08              | 0.67 ± 0.09              | 0.22 (12)                          |
| Glutamine            | 0.42 ± 0.02              | 0.75 ± 0.09              | 0.33 (18)                          |
| Asparagine           | 0.57 ± 0.04              | 1.60 ± 0.13              | 1.03 (55)                          |
| Alanine              | 0.37 ± 0.08              | 1.37 ± 0.21              | 1.01 (54)                          |
| Glutamate            | 0.59 ± 0.09              | 2.30 ± 0.11              | 1.71 (91)                          |
| Aspartate            | 0.68 ± 0.13              | 2.50 ± 0.25              | 1.83 (98)                          |
| Arginine             | 0.40 ± 0.11              | 2.64 ± 0.23              | 2.24 (120)                         |
| MeAIB                | 0.56 ± 0.12              | 2.76 ± 0.21              | 2.20 (118)                         |
| Leucine              | 0.31 ± 0.08              | 2.81 ± 0.53              | 2.50 (114)                         |
| Phenylalanine        | 0.46 ± 0.03              | 3.08 ± 0.45              | 2.62 (140)                         |

*a MeAIB, α-(methylamino)isobutyric acid.

because the Hill coefficient was significantly greater than 1 (1.4 ± 0.1) (Fig. 4D).

The reversal of the glutamine-induced currents when the membrane potential was depolarized beyond −20 to −30 mV as evident in Fig. 4B was interesting. Because this reversal potential was comparable to the equilibrium potential for Cl−, we wondered whether this could be due to the involvement of Cl− ions in the transport process. We therefore tested the influence of Cl− in the perfusion medium on glutamine-induced currents in human SN1-expressing oocytes. The currents and the current-voltage relationship were not significantly altered by the presence or absence of Cl− (data not shown). It thus appeared that the reversal potential was not due to Cl− movements. We then tested whether the phenomenon was unique to glutamine or was also seen with other substrates of SN1 as well. Surprisingly, the reversal of the currents was not observed with asparagine as the substrate (Fig. 5A). The asparagine-induced currents were, however, concentration-dependent (Fig. 5A) and pH-dependent (Fig. 5B) as was the case with the glutamine-induced currents. Additional experiments carried out with asparagine as the substrate showed that the transport function of human SN1 could be supported to a significant extent by Li+ in the place of Na+ (Fig. 6, A and B). The magnitude of currents induced by asparagine in the presence of Li+ was about 60% of the current induced in the presence of Na+. There was, however, no detectable current when the perifusion medium contained NMDG chloride in place of LiCl or NaCl. Kinetic analysis of the asparagine-induced currents was then carried out in the presence of Na+. The currents were saturable with increasing concentrations of asparagine at all membrane potentials tested (Fig. 5A). The K0.5 for asparagine was 16 ± 3 mM at −70 mV, and the value decreased significantly when the membrane was hyperpolarized beyond −70 mV. The K0.5 for asparagine was 11 ± 2 mM at −150 mV. Na+ activation kinetics of asparagine-induced currents showed a sigmoidal relationship (Fig. 7B). The value for the Hill coefficient was 1.4 ± 0.1 at −70 mV, suggesting involvement of more than one Na+ ion per transport cycle. The Hill coefficient did not change significantly under conditions of different membrane potentials (−10 to −150 mV).

The experiments described thus far show clearly that expression of the human SN1-expressing oocytes with SN1-specific substrates leads to induction of inward currents. To determine

**Fig. 2. pH-Dependence (A) and saturation kinetics (B) of human SN1-mediated glutamine transport.** A, transport of 50 μM [3H]glutamine was measured in vector-transfected cells (●) and in human SN1 cDNA-transfected cells (■) using the LiCl-containing medium in the presence of 10 mM cysteine. The pH of the medium was varied between 6.0 and 8.5 by appropriately adjusting the concentrations of Mes, Hepes, and Tris. B, glutamine transport was measured in vector-transfected cells and in human SN1 cDNA-transfected cells over a glutamine concentration range of 0.1–15 mM. The LiCl-containing medium (pH 8.5) with 10 mM cysteine was used in transport measurements. The cDNA-specific transport was calculated by subtracting the transport in vector-transfected cells from the transport in cDNA-transfected cells. These values for cDNA-specific transport were used in kinetic analysis. Inset, Eadie-Hofstee plot.

function electrophysiologically using the two-microelectrode voltage clamp technique.

When the oocytes expressing human SN1 were perifused with 5 mM glutamine in the presence of NaCl at pH 8.0, inward currents were detectable, implying that the transport process was associated with the transfer of net positive charge into the oocytes (Fig. 4A). The magnitude of the glutamine-induced currents was pH-dependent, decreasing as the pH of the perfusion medium was acidified. The currents were abolished completely at pH 5.5. Water-injected oocytes did not show glutamine-induced currents at any pH (data not shown). Because the inward currents are a measure of the transport activity of human SN1, the findings with regard to the marked pH dependence of human SN1 transport in X. laevis oocytes were similar to those found in mammalian cells. The magnitude of the glutamine-induced currents was also dependent on the concentration of glutamine and on the membrane potential (Fig. 4B). Kinetics analysis gave a K0.5 value (i.e. the concentration of glutamine yielding half-maximal currents) of 0.7 ± 0.1 mM (Fig. 4C). Na+ activation kinetics indicated that more than one Na+ was likely to be involved per transport cycle,
whether the induced inward currents are directly related to substrate influx into the oocytes, we measured the influx of asparagine, one of the substrates of SN1, and assessed the influence of K+-induced membrane depolarization on the influx (Fig. 8A). The substrate influx was measured with [3H]asparagine. The SN1-specific asparagine influx was inhibited 33% when the oocyte membrane was depolarized with high K+ in the extracellular medium (control, 193.6 ± 12.1 pmol/oocyte/h; membrane depolarization, 129.8 ± 11.8 pmol/oocyte/h; asparagine concentration, 250 μM). The inhibition of SN1-mediated amino acid influx by membrane depolarization indicates that the influx process is associated with transfer of positive charge into the oocyte, thus providing the basis for the inward currents that accompany the transport process. We also determined the ratio of substrate transfer to charge transfer in the same oocyte expressing human SN1 (Fig. 8B). The ratio was close to 1 (3 oocytes), indicating that the transfer of each molecule of amino acid substrate via human SN1 is accompanied by the transfer of one positive charge. These results show convincingly that the inward currents observed in SN1-expressing oocytes upon exposure to amino acid substrates are directly related to substrate influx.

**Electrogenicity of Rat SN1**—Our present results with human SN1 regarding the electrogenicity of the transport process are in contrast to the results with rat SN1 reported by Chaudhry et al. (19). To determine if these different results could be explained on the basis of species variations, we studied the transport function of rat SN1 in X. laevis oocytes using electrophysiological approaches. We isolated a full-length rat SN1 cDNA while screening a skeletal muscle cDNA library for SN1-related transporters. This was surprising to us, because Chaudhry et al. (19) reported that SN1 mRNA was not detectable in rat skeletal muscle. Based on the Northern blot analysis reported by these investigators, SN1 mRNA was detectable at high levels in the liver and at comparatively low levels in the kidney, heart, and brain. There were no detectable mRNA transcripts in the skeletal muscle, gut, lung, and spleen. However, when we screened a rat skeletal muscle cDNA library with the g17 cDNA fragment as the probe (the same probe used to isolate the full-length human SN1 cDNA from HepG2 cell cDNA library), several positive clones were identified and purified. One of these clones was found to code for a protein with an amino acid sequence identical to that of rat SN1. We expressed this clone in X. laevis oocytes and assessed its electrogenicity. As shown in Fig. 9A, perfusion of oocytes expressing rat SN1 with 10 mM asparagine at pH 8.0 in the presence of Na+ led to the induction of large inward currents. The magnitude of the current was highly pH-sensitive and decreased as the pH of the medium was made acidic. It was also Li+-tolerant to a significant extent (Fig. 9B). Replacement of Na+ with Li+ did not abolish asparagine-induced currents completely. The magnitude of the currents in the presence of Li+ was 30–40% of the magnitude of the currents detected in the presence of Na+. The Li+-supported currents were also pH-sensitive as the Na+-supported currents. These data show that rat SN1 is also electrogenic as is its human homolog.

**Genomic Organization of Human sn1 Gene**—A GenBank database search with the nucleotide sequence of human SN1 cDNA revealed that the human gene coding for this transporter has been sequenced in its entirety. The gene is located on chromosome 3p21.3 and is ~16 kbp long. By aligning the nucleotide sequence of the cloned human SN1 cDNA with the genomic sequence allowed us to deduce the exon-intron organization of the gene. The gene consists of 16 exons and 15 introns. The size of each of the exons and introns and the nucleotide sequences of the splice junctions are given in Table II. The 5′- and 3′-termini of each intron possess the consensus sequence for RNA splicing (gt/aga). The translation start site ATG is present in exon 2, and the translation termination site TGA is present in exon 16. Exon 1 does not code for the protein.

**DISCUSSION**

The studies reported here describe the cloning of a human amino acid transport system N (SN1) and the functional characteristics of the cloned transporter. Rat SN1 has recently been cloned from brain (19). This transport system is expressed most predominantly in the liver and, to a smaller extent, in the brain. Functional studies with rat SN1 have shown that SN1 is a Na+- and H+-coupled amino acid transporter with preference for glutamine, histidine, and asparagine (19). These three amino acids contain nitrogen in the side chain, a fact that led to the designation of the transporter as system N in the original studies describing the transport process responsible for the
uptake of these amino acids in hepatocytes (10). Rat SN1 mediates the influx of Na$^{+}$ and glutamine into the cells coupled to the efflux of H$^{+}$. In the present study, we cloned human SN1 from the HepG2 liver cell line. Human SN1, similar to the rat homolog, mediates the transport of glutamine in a Na$^{+}$- and H$^{+}$-coupled manner. Its substrate specificity is restricted to glutamine, histidine, asparagine, and alanine. However, glutamine and histidine are recognized by the transporter with a 5-fold greater affinity than asparagine and alanine. Human SN1 is Li$^{+}$-tolerant, because its transport function can be supported by a Li$^{+}$ gradient in place of a Na$^{+}$ gradient. There is no involvement of Cl$^{-}$ ions. The transporter shows marked pH dependence consistent with H$^{+}$ efflux in the transport process. The transporter is stimulated by an outwardly directed H$^{+}$ gradient. Thus, an inwardly directed Na$^{+}$ gradient and an
outwardly directed $H^{+}$ gradient provide the driving force for the transport process mediated by human SN1. This suggests that the transporter mediates the influx of $Na^{+}$ and glutamine into the cells coupled to the simultaneous efflux of $H^{+}$ out of the cells. These findings are similar to those described previously by Chaudhry et al. (19) for rat SN1.

The participation of an outwardly directed $H^{+}$ gradient as a driving force for SN1 may be related to our findings in the present study that the amino acids leucine and phenylalanine cause a significant stimulation of SN1-mediated glutamine transport. Leucine and phenylalanine are high affinity substrates for the amino acid transport system L that is known to be coupled to $H^{+}$ (35–37). An inwardly directed $H^{+}$ gradient stimulates system L activity, suggesting cotransport of $H^{+}$ and amino acid (37). The involvement of $H^{+}$ has also been demonstrated with one of the cloned subtypes of system L (38, 39). Therefore, it is possible that leucine and phenylalanine acidify intracellular pH as a result of their transport via system L in HRPE cells. Such an effect on the intracellular pH is expected to stimulate the transport activity of SN1.

The most important finding in the present study is that the transport process mediated by human SN1 is electrogenic. This is in contrast to the results of Chaudhry et al. (19), which showed the transport process mediated by rat SN1 to be electroneutral. We show here, however, that the differences between our studies and those by Chaudhry et al. (19) are not due to species variations between rat SN1 and human SN1 in the transport mechanism. We were able to demonstrate the electrogenicity of the transport process not only with human SN1 but also with rat SN1. The supporting evidence for the electrogenicity of SN1-mediated transport process comes from two different experimental approaches. When expressed in mamalian cells as well as in $X. laevis$ oocytes, human SN1 exhibits an $Na^{+}$:glutamine stoichiometry of 2:1. Considering the efflux of $H^{+}$ that is coupled to the transport process, this stoichiometry suggests that the overall transport mechanism is likely to be electrogenic, resulting in the transfer of a net positive charge into the cells. Unequivocal evidence for the electrogenic nature of SN1 was obtained in $X. laevis$ oocytes where glutamine (or asparagine) transport mediated by human or rat SN1 was found to be associated with inward currents detectable by the two-microelectrode voltage clamp technique. Inward currents under these experimental conditions mean that the transport process is associated with the transfer of net positive charge into the oocytes. The involvement of membrane potential in the SN1-mediated transport process was confirmed by the inhibition of SN1-mediated asparagine influx by membrane depolarization and by the establishment of the 1:1 ratio for the asparagine influx versus charge influx. Therefore, the membrane potential also plays a very important role in the
energizing of SN1 in addition to the transmembrane gradients for Na\(^+\) and H\(^+\).

There is no doubt that the identity of the driving forces involved in the transport function of SN1 is important to understand the transport mechanism. But, it is also relevant to the transport function of SN1 in the physiological context. Glutamine transport in the liver plays a significant role in urea synthesis, ammonia metabolism, and pH regulation (1). Interestingly, the direction of glutamine transport differs between the hepatocytes surrounding the portal veins and the hepatocytes surrounding the hepatic veins. Periportal hepatocytes take up glutamine and ammonia from the portal blood. Inside the cells, glutamine is hydrolyzed by glutaminase to liberate ammonia. The glutamine-derived ammonia as well as ammonia taken up from the portal blood are converted into urea. Thus, periportal hepatocytes express glutaminase and urea cycle enzymes and also a transport system responsible for the influx of glutamine. In contrast, perivenous hepatocytes take up ammonia from venous blood and use it to synthesize glutamine rather than urea. The synthesized glutamine is released into venous blood. Thus, perivenous hepatocytes express glutamine synthetase but very little urea cycle enzymes. In addition, these hepatocytes express a transport system responsible for the efflux of glutamine. Interestingly, studies by Chaudhry et al. (19) have shown that SN1 is expressed uniformly in periportal hepatocytes as well as in perivenous hepatocytes. This suggests that SN1 may mediate the influx of glutamine in periportal hepatocytes and the efflux of glutamine in perivenous hepatocytes. The shift in the function of SN1 from a mediator of glutamine influx in periportal hepatocytes to a mediator of glutamine efflux in perivenous hepatocytes is expected to be intimately involved with changes in driving forces that energize the transport process. The transmembrane H\(^+\) gradient and the transmembrane glutamine gradient appear to be important in this context. Because periportal hepatocytes synthesize urea, which requires not only ammonia but also HCO\(_3^−\), these cells have an effective means of HCO\(_2^−\) disposal. In contrast, perivenous hepatocytes remove ammonia via glutamine synthesis rather than urea synthesis, and this process does not involve HCO\(_2^−\). Thus, perivenous hepatocytes do not have an effective means of HCO\(_2^−\) disposal. Therefore, it is likely that the intracellular pH in periportal hepatocytes is acidic compared with the intracellular pH in perivenous hepatocytes. An acidic intracellular pH in periportal hepatocytes is expected to facilitate the transport function of SN1 in the direction of glutamine influx. Similarly, an alkaline intracellular pH in perivenous hepatocytes is expected to facilitate the transport function of SN1 in the direction of glutamine influx. In addition, the transmembrane glutamine gradient differs significantly between periportal and perivenous hepatocytes. Periportal hepatocytes possess glutaminase that effectively hydrolyzes glutamine, thus maintaining low intracellular levels of glutamine. On the other hand, perivenous hepatocytes possess glutamine synthetase that generates glutamine, thus maintaining relatively high intracellular levels to glutamine. These conditions favor glutamine influx via SN1 in periportal hepatocytes and glutamine efflux via SN1 in perivenous hepatocytes. This hypothesis is supported by in vitro liver perfusion studies (1, 40). Lowering the perfusate pH decreases urea production from portal ammonia and glutamine and increases glutamine production. Increasing the perfusate pH has opposite effects. It enhances urea production from portal ammonia and glutamine and decreases glutamine production. These findings support the hypothesis, because lowering the perfusate pH is expected to decrease glutamine influx via SN1 in urea-producing periportal hepatocytes and increase glutamine efflux via SN1 in glutamine-producing perivenous hepatocytes. Increasing the perfusate pH has the opposite effects. There is no information available on whether there are any significant differences in membrane potential between periportal hepatocytes and perivenous hepatocytes and associated with their distinct metabolic functions. Such information may be useful, because the magnitude of membrane potential also has a role in determining the direction of glutamine flux via SN1.

The ability of SN1 to mediate the influx or efflux of glutamate depending upon the magnitude and direction of the driving forces provides a possible explanation for the results of glutamine transport obtained in vector-transfected cells under different experimental conditions. Obviously, whether or not the transport function of the heterologously expressed SN1 becomes detectable in HRPE cells depends on the endogenous glutamine transport activity. If the endogenous activity is high as evident when measured in a NaCl-containing medium, SN1 actually mediates the efflux of glutamine. This is because the endogenous activity increases the intracellular levels of glutamine high enough to make the heterologously expressed SN1 operate in the efflux mode. This explains why the glutamine accumulation in SN1 cDNA-transfected cells is lower than in control cells. However, when the transport of glutamate via endogenous transport activities is specifically and effectively blocked by replacing NaCl with LiCl and by including cysteine in the medium, conditions that do not interfere with the transport function of SN1, the heterologously expressed SN1 is able to mediate the influx of glutamate. Thus, the transport activity...
of SN1 as measured by glutamine influx becomes easily detectable under these conditions.

Another interesting and physiologically relevant finding from the present study is that SN1 is expressed in skeletal muscle. Even though the studies by Chaudhry et al. (19) failed to detect SN1 mRNA in rat skeletal muscle, the screening of a rat skeletal muscle cDNA library in our study has led to the isolation of a full-length SN1 cDNA that is functional in heterologous expression systems. We screened the library in an attempt to clone system Nm, a variant of system N that is expressed specifically in skeletal muscle (11). Based on the data reported by Chaudhry et al. (19), we were not expecting to find SN1 cDNA in the library. Quite contrary to our expectation, the screening of the library led to the isolation of SN1 from skeletal muscle. Our present results clearly show that SN1 is expressed in skeletal muscle. We have not yet succeeded in identifying a clone from the library with functional characteristics of Nm. The identification of SN1 expression in skeletal muscle is important because of the well recognized role of glutamine transport in this tissue in various physiological states (41). There is evidence for glutamine influx as well as glutamine efflux in skeletal muscle depending on the physiological state. In the postabsorptive state, glutamine is released from skeletal muscle, thus supplying an important metabolic fuel to the rapidly dividing cells of the intestine and immune system. Glutamine and alanine together make up 80% of the total amino acids released from this tissue (7, 42, 43). Glutamine efflux in skeletal muscle depending on the physiological states (41). There is evidence for glutamine influx as well as glutamine efflux in skeletal muscle depending on the physiological state. In the postabsorptive state, glutamine is released from skeletal muscle, thus supplying an important metabolic fuel to the rapidly dividing cells of the intestine and immune system. Glutamine and alanine together make up 80% of the total amino acids released from this tissue (7, 42, 43).

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