High affinity choline uptake plays a critical role in the regulation of acetylcholine synthesis in cholinergic neurons. Recently, we succeeded in molecular cloning of the high affinity choline transporter (CHT1), which is specifically expressed in cholinergic neurons. Here we demonstrate the presence of functionally relevant, nonsynonymous single nucleotide polymorphism in the human CHT1 gene by comprehensive sequencing of the coding region and the intron/exon boundaries including the transcription start site. The deduced amino acid change for the polymorphism is isoleucine to valine at amino acid 89 (I89V) located within the third transmembrane domain of the protein. The allele frequency of I89V was 6% for Ashkenazi Jews. Functional assessment of the I89V transporter in mammalian cell lines revealed a 40–50% decrease in V_{max} for choline uptake rate compared with the wild type, whereas there was no alteration in the apparent affinities for choline, sodium, chloride, and the specific inhibitor hemicholinium-3. There also was no change in the specific hemicholinium-3 binding activity. The decreased choline uptake was not associated with the surface expression level of the protein as assessed by biotinylation assay. These results suggest an impaired substrate translocation in the I89V transporter. The Caenorhabditis elegans ortholog of CHT1 has a valine residue at the corresponding position and a single replacement from valine to isoleucine caused a decrease in the choline uptake rate by 40%, suggesting that this hydrophobic residue is generally critical in the choline transport rate in CHT1. This polymorphism in the allelic CHT1 gene may represent a predisposing factor for cholinergic dysfunction.

In the peripheral and central nervous system, cholinergic neurons are involved in diverse behavioral and mental functions. In the cholinergic neurons, choline is taken up into the presynaptic terminals by the Na^+-dependent, high affinity choline transporter and subsequently used for acetylcholine (ACh) synthesis catalyzed by the enzyme choline acetyltransferase (ChAT) (1, 2). ACh is subsequently transported into synaptic vesicles by the vesicular acetylcholine transporter (2, 3). Choline uptake is thought be the rate-limiting step in ACh synthesis, because the rate of ACh synthesis correlates well with that of choline uptake (1, 4–7). ChAT is present in a kinetic excess and is not thought to be the rate-limiting factor for ACh synthesis (1, 7). The high affinity choline uptake is acutely regulated by neuronal activity (7, 8), and so it could be a unique marker for cholinergic neuronal activity as well as a marker of cholinergic neurons. The high affinity choline uptake is selectively inhibited by hemicholinium-3 (9).

Recently, we succeeded in functional cloning of the high affinity choline transporter (CHT1) from the nematode Caenorhabditis elegans using information provided by the C. elegans Genome Project (10), and successively cloned the orthologs from rat (10) and human (11). CHT1 was found to belong to the Na^+/solute symporter family (12) (TC 2.A.21 in NCBI). The members of the family utilize the Na^+ electrochemical gradient to drive the coupled transport of a variety of substrates (glucose, amino acids, osmolytes, myo-inositol, iodide, and vitamins). When expressed heterologously, CHT1 showed Na^+- and Cl^−-dependent high affinity choline uptake activity that is highly sensitive to hemicholinium-3 at nanomolar concentrations. CHT1 mRNA is expressed exclusively in cholinergic neurons. Very recent immunohistochemical studies revealed that the CHT1 protein was expressed specifically in cholinergic neurons including their terminals in the central nervous system of rat (13, 14) and primates (15). Molecular identification of CHT1 enabled homology-based molecular cloning and characterization of CHT1 orthologs from various species including human (16), Limulus (17), mouse (18), and Torpedo (19). These results indicate that CHT1 represents the high affinity choline transporter expressed specifically in cholinergic neurons from vertebrate to invertebrate.

Cholinergic neurons are vital for motor, autonomic, and cognitive functions and are implicated in the pathophysiology of Alzheimer's disease (20, 21), Down's syndrome (21), Parkinson's disease (22), and schizophrenia (23).
son’s disease (22), and schizophrenia (23). In Alzheimer’s disease, brain cholinergic deficits were recognized, as shown by decrease in ChAT activity with compensatory increases in the high affinity choline transporter (24, 25). These results suggest the possibility that mutation(s) or polymorphism(s) in CHT1 may act as associated factors or predisposing factors for these diseases or as modifiers of the disease phenotypes. The other members of the Na+/glucose transporter (SGLT1) in glucose/galactose malabsorption (26, 27) and the Na+/H+ symporter (NIS) in congenital hypothyroidism (28).

We therefore screened for a polymorphism within the entire translated region of the human CHT1 gene in an Ashkenazi Jewish population and identified only one nonsynonymous polymorphism that predicts the amino acid substitution in the transporter protein. The substitution occurs in the putative third transmembrane region and was expected to affect the choline transporter function. We analyzed the functional properties of each transporter by expressing it in mammalian cell lines and demonstrated that the mutant transporter is functional, but exhibits altered choline transport rate.

**EXPERIMENTAL PROCEDURES**

**Determination of the Transcription Start Site**—The 5′-terminal sequence of the hCHT1 cDNA from human spinal cord mRNA (Clontech) was determined using the FirstChoice™ RLM-RACE kit (Ambion) based on the oligo-capping cloning method (29), following the manufacturer’s protocol. Briefly, the PCR template was synthesized with reverse transcriptase and the hCHT1 gene-specific primer (5′-AGCTATGACTGTGGGAAAGACG-3′) from human spinal cord mRNA, 5′-capped with an oligo-RNA adaptor (5′-GGCAGUGGUGGAGUGACAC-3′) from human spinal cord mRNA, and reverse primer (5′-GCCTTTGATG-3′). The nested PCR reactions were performed with cap adaptor primers (5′-CGTATGATTGC-3′, 5′-CAGCTCTCACTTGACG-3′, and 5′-GGCAGUGGUGGAGUGACAC-3′). The reactions were performed using the following conditions: 95°C for 1 min and then 5 cycles of 95°C for 30 s and 68°C for 1 min, and 30 cycles of 95°C for 30 s, 65°C for 30 s, and 68°C for 1 min, with a final elongation of 68°C for 1 min. PCR products were subcloned and sequenced. The transcription start site was determined by identification of the boundary sequence between the RNA adapter and hCHT1 mRNA sequence.

**Polymorphism Detection**—The human CHT1 gene (GenBank™ accession number AC009963 and AC023672) from genomic DNA was amplified by PCR. Human DNA samples from unrelated Ashkenazi Jewish donors were obtained from the National Laboratory for the Genetics of Israeli Populations at Tel-Aviv University in Israel. The 1740-bp coding region as well as 291-bp 5′-untranslated region (UTR) and 573-bp 3′-UTR were examined. As an initial examination, DNA from 57 individuals was utilized. Separate PCR reactions were performed on each DNA sample to amplify the 9 exons of the hCHT1 gene and the proximal promoter region. Specific primer sets were designed based on the nucleotide sequence of the hCHT1 gene (Table I). PCR was carried out using ~10 ng of genomic DNA, consisting of dNTPs (0.2 mM each), the specific primer pair (0.2 μM each), PCR buffer (PerkinElmer Life Sciences) with 2.5 unit of AmpliTag™ Gold DNA Polymerase (PerkinElmer Life Sciences), and a total reaction volume of 25 μl. Reactions were started by an initial incubation at 95°C for 10 min and followed by 5 cycles of 95°C for 30 s, 68°C for 1 min, and 72°C for 30 s, 5 cycles of 95°C for 30 s, 64°C for 1 min, and 72°C for 30 s, and 25 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 30 s. Direct sequencing of both strands of each PCR product was performed by an Applied Biosystems sequencer using dye terminator methods. The adenine of the initiator ATG codon was designated as nucleotide 1, and amino acid 1 was the encoded methionine. An A to G transition at nucleotide 265 that resulted in a change from isoleucine to valine at amino acid 89 was identified (see Fig. 2). Because this nucleotide change resulted in loss of a unique MunI restriction endonuclease site in the PCR fragment, the presence or absence of this polymorphism in additional samples was studied by MunI digestion of the fragment of the PCR product (Fig. 2D).

**Site-directed Mutagenesis**—Point mutations were introduced into the wild-type hCHT1 and the wild-type C. elegans CHO-1 cDNAs by PCR using oligonucleotide primers encoding the mutations. The following mutations were introduced: hCHT1 I89V, I89A, and I89F, and C. elegans CHO-1 V90I. Identity of the mutant cDNAs was confirmed by sequencing.

**[3H]Choline Uptake Assay**—To assess transport activity of hCHT1 and the hCHT1 Ile89-mutants, as well as the C. elegans CHO-1 and its mutant, the transporters were transiently expressed in COS-7 cells or HEK293 (tsA-201) cells. hCHT1 and C. elegans CHO-1 cDNA were subcloned into the HindIII/BamHI sites of the vector pcDNA3.1(+) (Invitrogen). COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, at 37°C in 5% CO2 atmosphere. HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium/ F-12 supplemented with 10% fetal bovine serum. For uptake experiments, ~90% confluent cells in 100-mm dishes were transfected with cDNA using Opti-MEM (Invitrogen) and LipofectAMINE 2000 reagent (Invitrogen), plated in 24-well culture plates the following day, and used for uptake assays 48 h later. Cells were washed with Krebs-Ringer’s HEPES (KRH) buffer (130 mM NaCl, 1.3 mM KCl, 2.2 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 10 mM HEPES, 10 mM glucose, pH 7.4) and preincubated for 1–2 h at 37°C in KRH buffer for equilibration, followed by an additional preincubation with KRH buffer for 10 min. Uptake assays with 0.01–0.1 μM [3H]choline (82.0 Ci/mmol; Amersham Biosciences) were performed for 5 min at 37°C or at room temperature. Specific inhibitor, hemicholinium-3 (HC-3, 1 μM), was added in the preincubation step. Saturation kinetics were determined using increasing concentrations of [3H]choline with the specific activity diluted with unlabeled choline. Uptake was terminated by three washes with ice-cold KRH buffer. The level of accumulated [3H]choline was determined by solubilizing cells in 1% SDS, 0.2% NaOH and then doing a liquid scintillation count. The Na+ dependence of [3H]choline uptake was assessed in Buffer A (140 mM NaCl, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 5 mM Tris, 10 mM glucose, pH 7.4) using isotonic replacement of NaCl with LiCl. The Cl− dependence was determined in Buffer A with Cl− salts replaced by sodium gluconate, potassium gluconate, and calcium nitrate at molarities equivalent to those in the original Buffer A. Specific [3H]choline uptake was determined by subtracting the uptake in the presence of 1 μM HC-3 from the total uptake, or by subtracting the uptake for cells transfected with the pcDNA3.1 alone from the uptake for cells transfected with the cDNA. Km for choline or HC-3 were calculated by nonlinear least-square fits (KaleidaGraph, Synergy Software) using the Hill equation. Assays were performed in triplicate or quadruplicate and repeated in two or three separate experiments.

**[3H]Hemicholinium-3 Binding Assay**—HC-3 binding experiments using [3H]HC-3 (PerkinElmer Life Sciences, 128 Ci/mmol) were performed using intact cells. Forty-eight hours after transfection, cells were
washed with KRH buffer and preincubated for 1–2 h at 37 °C for equilibrium. Binding assays were performed at 4 °C for 1–2 h and terminated by three washes with ice-cold KRH buffer. Saturation binding was determined in triplicate using 0.1–10 nM [3H]HC-3 with or without 1 μM nonlabeled HC-3 used to determine nonspecific binding.

**Immunofluorescent Analysis—** COS-7 cells were transfected with cDNA encoding wild-type hCHT1, 189V hCHT1, or plasmid alone and plated on a 4-well slide chamber (Nunc) on a glass substrate. Cells were fixed in 4% paraformaldehyde in PBS for 20 min, permeabilized with 0.2% Triton X-100 in PBS, and blocked with PBS containing 2% normal goat serum. Fixed cells were incubated overnight with affinity purified anti-choline antibody (1:1000) (15) in PBS containing 2% normal goat serum at 4 °C, and then with the secondary antibody (Alexa Fluor 488-conjugated goat anti-rabbit IgG; Molecular Probes, Eugene, OR) for 1 h (1:1000) in PBS. Cells were washed three times with PBS and then formatted with a confocal microscope (Bio-Rad). Images obtained with Bio-Rad software were then formatted in Adobe Photoshop software.

**Cell Surface Biotinylation Assay—** To determine expression levels of the wild-type and 189V hCHT1 on the cell surface, biotinylation assays using affinity purified anti-CHT1 antibody were performed. [3H]HC-3 binding assays were performed simultaneously with biotinylation assays. The cDNAs were transiently expressed in HEK293 cells. Forty-eight hours after transfection, cells were preincubated for 1 h at 37 °C in KRH buffer, followed by rinsing with ice-cold PBS/calcium-magnesium (138 mM NaCl, 2.7 mM KCl, 1.5 mM KHPO₄, 9.6 mM Na₂HPO₄, 0.1 mM CaCl₂, 1 mM MgCl₂, pH 7.4). Cells were treated with Sulfo-NHS-S-S-Biotin (1 mg/ml, Pierce) in PBS/calcium-magnesium at 4 °C for 30 min. Unreacted biotin was quenched by washing with 100 mM glycine in PBS/calcium-magnesium twice and incubating with the same buffer for 45 min. Cells were solubilized with radioimmunoprecipitation assay buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton-X-100, 1% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (Complete; Roche Molecular Biochemicals) at 4 °C. Cell lysates were centrifuged at 17,000 × g for 30 min at 4 °C to sediment out nucleic acids and debris, and protein concentrations were determined by the BCA method (Pierce). Biotinylated proteins were separated from nonbiotinylated proteins by incubation with UltraLinkTM Plus Immobilized Streptavidin (Pierce). Biotinylated proteins were separated from nonbiotinylated proteins by incubation with UltraLinkTM Plus Immobilized Streptavidin (Pierce). Biotinylated proteins were separated from nonbiotinylated proteins by incubation with UltraLinkTM Plus Immobilized Streptavidin (Pierce).

Statistical differences were determined by Student's t test for two-group comparisons.

**RESULTS**

To identify the 5'-end of the human CHT1 (hCHT1) gene, we determined the transcription start site of the hCHT1 gene by 5'-rapid amplification of cDNA ends (5'-RACE) using human spinal cord mRNA and nested hCHT1 gene-specific primers. Gel electrophoresis of RACE products showed a single band with a length of ~300 bp, which was absent from the product from mRNA pretreated without tobacco acid pyrophosphatase, suggesting that this band was specifically amplified from the full-length, capped mRNA (Fig. 1). Sequence analysis of the ~300-bp RACE product revealed that the known mRNA sequence (GenBankTM accession number AB043397) was elongated towards the 5'-end by 15 bp. The 5'-UTR of the hCHT1 mRNA is 291 bp in length (GenBankTM accession number AB084377). There is a TATA box in the proximal hCHT1 5'-flanking region located at position −30 relative to the transcription start site, and a putative Sp1 binding site at position −37.

The entire hCHT1 gene is ~25 kb in length with nine exons and eight introns. We comprehensively sequenced all the exons (except the region corresponding to 3'-UTR) and the intron/exon boundaries including the transcription start site of the hCHT1 gene from genomic DNA of 57 Ashkenazi Jews. Sequence analysis of the entire coding region as well as 5'-UTR revealed only one single nucleotide polymorphism. The nonsynonymous polymorphism consisted of an A to G transition at nucleotide 265 (A265G, Fig. 2, A–D). The deduced amino acid change for this polymorphism is isoleucine to valine at amino acid 89 (189V), located within the third transmembrane domain of the protein. Seven of the 57 chromosomes showed heterozygous alleles (A/G) and the allele frequency of this A265G was estimated to be 0.06.

To assess the consequences of this polymorphism on transporter function, we transiently transfected COS-7 cells with cDNA encoding either the wild-type (WT) or the 189V variant hCHT1 and measured [3H]choline uptake activity. Expression of 189V resulted in ~40% reduction in choline uptake compared with WT (Fig. 3A). Kinetic analysis indicated that 189V showed ~40% reduction in $V_{max}$ with no significant change in the apparent affinity for choline (Fig. 3B and Table II). The $K_m$ value for choline was 2.8 ± 0.1 μM for WT and 3.0 ± 0.3 μM for 189V ($n = 4$). A similar difference in $V_{max}$ values was also observed for WT and 189V expressed in HEK293 cells (data not shown). [3H]HC-3 binding assays for WT and 189V revealed essentially the same dissociation constants ($K_d$ ~6 μM) and binding capacities for HC-3 (Fig. 3C and Table II). Consistent with this finding, the choline uptake was inhibited by HC-3 with essentially the same $K_i$ of ~2 μM in both WT and 189V (Fig. 3D). Because HC-3 is hydrophilic and impermeable to cell membrane, the HC-3 binding activity represents the expression level of the transporter in the plasma membrane. Thus, the same binding capacities for HC-3 indicate that the expression of hCHT1 in the plasma membrane is not significantly affected by the replacement of Ile by Val at amino acid 89. Overall, these results indicate that the 189V mutation in hCHT1 affects the choline uptake activity but not the apparent affinity for choline and HC-3 (Table II).

Substitution of Li⁺ for Na⁺, or gluconate for Cl⁻ resulted in almost full loss of choline uptake for either WT-transfected or 189V-transfected cells (data not shown). When Na⁺ was partly replaced by Li⁺, WT and 189V exhibited a similar dependence on Na⁺ concentration (Fig. 4A). hCHT1 showed a sigmoidal...
dependence on Na$^+$ concentration, as described previously (11). Na$^+$ dependence for both WT and I89V did not become saturated in an isotonic substitution range, and so we could only estimate the $K_{m}$ value as >100 mM. When we replaced Cl$^-$ with gluconate, the Cl$^-$ dependence of hCHT1 was not affected by the I89V mutation. Cl$^-$ dependence of choline uptake could be fit to a single hyperbola for both WT and I89V, suggesting a stoichiometry of one Cl$^-$ per one choline molecule (Fig. 4B). This coupling stoichiometry is in contrast to our previous result, in which NaCl was replaced by NaI and the choline uptake activity showed a sigmoidal dependence on Cl$^-$ concentration (11). Iodide might have exerted an inhibitory effect on the uptake activity rather than acting as an inert anion, as observed in pantothenate uptake by Na$^+$-dependent multivitamin transporter (30). This 1 Cl$^-$/1 choline coupling stoichiometry agrees with the result from high affinity choline uptake in brain synaptosomes, in which Cl$^-$ was replaced by acetate (31). Cl$^-$-dependent uptake by WT and I89V were saturated in the investigated substitution range. When fitting the Cl$^-$ dependence of choline uptake to the Hill equation, the apparent affinity for Cl$^-$ was $21 \pm 2$ mM ($n = 3$; nHill = 1.1 $\pm$ 0.1) for WT and $22 \pm 3$ mM ($n = 3$; nHill = 1.0 $\pm$ 0.1) for I89V (Fig. 4B).

Indirect immunofluorescence, in combination with confocal laser-scanning microscopy of hCHT1-transfected COS-7 cells, showed cytoplasmic punctate immunoreactivity for either WT or I89V (Fig. 5A). The staining patterns suggested that most of the proteins were retained in the cytoplasmic compartments and inefficiently targeted the plasma membrane. No staining was observed for mock-transfected cells (data not shown).

Direct immunoblots of WT- and I89V-transfected HEK293 cells showed no significant differences in expression levels of immunoreactive proteins (Fig. 5B, Total). CHT1 reactive proteins in cells were detected as major bands of ~45 and ~80 kDa for both WT and I89V, whereas these bands were absent from mock-transfected cells (Fig. 5B). The predicted molecular weight from the hCHT1 primary structure is 63,162. The various bands may represent oligomers and/or heterogeneous glycosylation of hCHT1 protein. The unaltered expression level of the I89V protein in the plasma membrane, as suggested by the result from the HC-3 binding assay, was confirmed by selective labeling of the transporters at the cell surface using a membrane-impermeable biotin analog. Biotinylated proteins were separated from nonbiotinylated proteins by incubation with streptavidin gel, and both proteins were subjected to Western analysis. Biotinylated proteins were thought to be mostly expressed on the cell surface, because they were greatly, although not completely, depleted of the intracellular marker actin (Fig. 5C). When CHT1 immunoreactivity in each biotinylated fraction was normalized with respect to the amount of actin immunoreactivity in total lysate fraction, no significant change was observed in I89V surface expression levels as compared with WT, supporting the result from the HC-3 binding assay, was confirmed by selective labeling of the transporters at the cell surface using a membrane-impermeable biotin analog. Biotinylated proteins were separated from nonbiotinylated proteins by incubation with streptavidin gel, and both proteins were subjected to Western analysis. Biotinylated proteins were thought to be mostly expressed on the cell surface, because they were greatly, although not completely, depleted of the intracellular marker actin (Fig. 5C). When CHT1 immunoreactivity in each biotinylated fraction was normalized with respect to the amount of actin immunoreactivity in total lysate fraction, no significant change was observed in I89V surface expression levels as compared with WT, supporting the result from the HC-3 binding assay that the expression levels of WT and I89V in the plasma membrane were not significantly different from each other.

The I89V polymorphism occurs in the putative third transmembrane region of hCHT1. Alignment of the CHT1 amino acid sequence from various species shows that the putative transmembrane domain is highly conserved, and that residue
89 is occupied by hydrophobic amino acid residues such as Ile, Phe, or Val (Fig. 6A). To confirm whether this conservative residue plays a critical role in choline transporter function, we introduced several mutations at this position (Ile-89) in hCHT1. As shown in Fig. 6, B and C, the substitution of Ala for Ile greatly reduced the choline uptake activity whereas the expression level in the plasma membrane was not greatly altered as assessed by the \( [^3H]Hemicholinium-3 \) binding activity and the apparent affinity for HC-3 was increased \( (K_d, 2\text{ nM for I89A versus } 6\text{ nM for WT}) \). The substitution of Phe for Ile reduced both the choline uptake and the \( [^3H]Hemicholinium-3 \) binding activity. The \( C.\ elegans \) ortholog of CHT1 (CHO-1) (10) has a valine residue at the corresponding position (amino acid 90, Val-90) (Fig. 6A). Accordingly, we next introduced isoleucine in place of valine at

<table>
<thead>
<tr>
<th>Transporter</th>
<th>( V_{max} ) pmol/min/mg protein</th>
<th>( K_m ) ( \mu M )</th>
<th>Hemicholinium-3 ( K_d ) ( nM )</th>
<th>( B_{max} ) fmol/mg protein</th>
<th>( K_d ) ( nM )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type CHT1</td>
<td>102 ± 9</td>
<td>2.8 ± 0.1</td>
<td>1.8 ± 0.4</td>
<td>118 ± 15</td>
<td>5.9 ± 1.0</td>
</tr>
<tr>
<td>I89V variant CHT1</td>
<td>55 ± 6*</td>
<td>3.0 ± 0.3</td>
<td>2.1 ± 0.5</td>
<td>108 ± 16</td>
<td>6.4 ± 0.6</td>
</tr>
</tbody>
</table>

* \( p < 0.01 \), compared with the wild-type CHT1 value, \( n = 4 \).
this position (V90I) in CHO-1. When expressed in HEK293 cells, the V90I transporter showed a 40% decrease in choline uptake with unaltered affinity for choline and essentially the same [3H]HC-3 binding activity (Fig. 6, D and E). These results indicate that this hydrophobic residue within the third transmembrane domain is, in general, critical in transport function of the high affinity choline transporter.

**DISCUSSION**

To date, to the best of our knowledge, no report has been available regarding the presence or functional relevance of genetic polymorphisms in the human high affinity choline transporter gene. In this report, we describe the identification of nonsynonymous single nucleotide polymorphism in the coding region of hCHT1 gene. Single nucleotide polymorphisms are increasingly recognized as a source of genetic variation, and their density may be as high as 1 per kb of cDNA (32). Most of these polymorphisms have a neutral effect on function, but some contribute to impaired protein function. Our analysis indicates that the I89V mutation does not affect the affinities for choline, Na\(^+\), Cl\(^-\), and HC-3. The unaffected potency of inhibitor as well as the unaffected \(K_m\) value for choline in I89V indicates that the mutation does not perturb the hCHT1 structure for ligand binding. In contrast, we found substantial functional differences between WT and I89V regarding the maximal transport capacity: the \(V_{\text{max}}\) value was 40% lower for I89V than for WT. This \(V_{\text{max}}\) reduction does not appear to be because of reduced translation of the protein or its reduced translocation to the cell surface, because the expression level of I89V in
the plasma membrane was essentially the same as that of WT as assessed by both the HC-3 binding activity and the cell surface biotinylation experiments in the intact cells. Thus this reduction in $V_{\text{max}}$ means that the translocation rate for choline bound to the I89V transporter is \(\frac{40}{100}\%\) lower than that for choline bound to the WT transporter. The functional significance of the Ile-89 residue in hCHT1 and the corresponding Val-90 residue in the C. elegans CHO-1 was confirmed by mutation studies of these residues with other amino acid residues.

The Ile-89 residue may be located either within the second or third transmembrane domain, depending on the topological model of CHT1, which is 12 or 13 transmembrane domains. We previously adopted the model with 12 transmembrane domains (10, 11), whereas other groups used a model with 13 transmembrane domains (16, 17). Although conclusive evidence for the topology with 12 or 13 transmembrane domains is lacking at present, the following circumstantial evidence appears to favor the model with 13 transmembrane domains. CHT1 has homology with members of the Na$^+$/H$^+$/solute symporter family, and a member of this family, the Na$^+$/glucose transporter, was shown to have an extracellular N terminus and an intracellular C terminus, indicating the presence of 13 transmembrane do-

---

**Fig. 6. Functional properties of various mutants of CHT1 in transiently transfected COS-7 or HEK293 cells.**

A, alignment of amino acid residues in the third transmembrane domain of CHT1 from various species. B, choline uptake mediated by WT, I89A, and I89F. C, $[^3H]$HC-3 binding using intact cells expressing WT, I89A, and I89F. D, choline uptake mediated by C. elegans CHO-1 WT and V90I mutant transporter. E, $[^3H]$HC-3 binding using intact cells expressing CHO-1 WT and V90I.
Our study represents the first detailed examination of genetic polymorphism of hCHT1 with a substitution of Val for Ile in the third transmembrane domain. The presence of functionally relevant polymorphic hCHT1 may account for interindividual variability in physiological responses where cholinergic neurons play a role. In addition, the hCHT1 polymorphism could be responsible for interindividual variability in disease predisposition or modification. Our findings suggest that at least some of the interindividual variation in the high affinity choline uptake activity could be explained by the genetic polymorphism at amino acid position 89 in the hCHT1 molecule. Under conditions in which choline uptake is the rate-limiting step in ACh synthesis, the lower activity of ChAT are associated with apnea in human (36). In this disease, mutations impairing nicotinic syndrome associated with frequently fatal episodes of apnea in human (36). In this disease, mutations impairing catalytic efficiencies of ChAT are associated with a stimulation-depolarization. Given that the cytoplasmic concentration of ACh in cholinergic nerve terminals of mammalian brain is estimated to be 0.2 to 1 mM (3) and that the vesicular acetylcholine transporter shows ACh uptake with a $K_m$ of $\approx 1$ mM (34), the rate of ACh uptake into synaptic vesicles may not be saturated in vivo. Indeed, overexpression of the vesicular acetylcholine transporter in *Xenopus* motor neurons results in an increase in the amplitude and frequency of miniature excitatory postsynaptic currents at the neuromuscular synapses (35). The cytoplasmic concentration of ACh in nerve terminals may be critical for the vesicular storage and release of ACh in vivo. Recently, mutations in chAT have been shown to cause a congenital myasthenic syndrome associated with frequently fatal episodes of apnea in human (36). In this disease, mutations impairing catalytic efficiencies of ChAT are associated with a stimulation-dependent decrease of the miniature end plate potential amplitude of the neuromuscular junction, suggesting that a defect in ACh synthesis has a significant impact on motor function. Considering the role of CHT1 in ACh synthesis, it is of interest to examine whether the hCHT1 polymorphism associated with impaired transport function in vivo has any consequences in vivo.

In conclusion, we report the identification and functional characterization of a single nucleotide polymorphism in the hCHT1 gene. We delineated the phenotypes of the polymorphism of hCHT1 with a substitution of Val for Ile in the third transmembrane domain. This naturally occurring mutation caused a significant reduction in the choline transport rate. Our study represents the first detailed examination of genetic polymorphism in the hCHT1 gene and creates a framework for further investigations of the consequences of hCHT1 polymorphisms in vivo.

**REFERENCES**

Single Nucleotide Polymorphism of the Human High Affinity Choline Transporter Alters Transport Rate
Takashi Okuda, Michiko Okamura, China Kaitsuka, Tatsuya Haga and David Gurwitz

doi: 10.1074/jbc.M207742200 originally published online September 16, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M207742200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 36 references, 7 of which can be accessed free at
http://www.jbc.org/content/277/47/45315.full.html#ref-list-1