Nuclear Choline Acetyltransferase Activates Transcription of a High-affinity Choline Transporter*§

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Choline acetyltransferase (ChAT) synthesizes the neurotransmitter, acetylcholine, at cholinergic nerve terminals. ChAT contains nuclear localization signals and is also localized in the nuclei of neural and non-neuronal cells. Nuclear ChAT might have an as yet unidentified function, such as transcriptional regulation. In this study, we investigated the alteration of candidate gene transcription by ChAT. We chose high affinity choline transporter (CHT1) and vesicular acetylcholine transporter (VACHT) as candidate genes, which function together with ChAT in acetylcholine production. Using SH-SY5Y human neuroblastoma cells stably expressing wild-type human ChAT, we found that overexpressed ChAT enhanced transcription of the CHT1 gene but not the VACHT gene. In contrast, nuclear localization signal disrupted, and catalytically inactive mutant ChATs could not induce, CHT1 expression. Additionally, ChAT did not alter CHT1 expression in non-neuronal HEK293 cells. Our results suggest that ChAT activates the transcription of selected target genes in neuronal cells. Both enzymatic activity and nuclear translocation of ChAT are required for its transcriptional enhancement.

Choline acetyltransferase (ChAT) catalyzes acetylation of choline to synthesize a neurotransmitter acetylcholine (ACh), which mediates neuronal transmission in both the peripheral and central nervous systems. Cholinergic nervous systems are involved in neuromuscular transmission and in the central cholinergic projection implicated in cognitive function. Several mutations of the CHAT gene have been reported to cosegregate with congenital myasthenic syndrome with episodic apnea (1). Moreover, cholinergic neurons are preferentially affected in several neurodegenerative diseases such as Alzheimer disease and amyotrophic lateral sclerosis (2).

The major isoform of human ChAT is a 69-kDa soluble protein, referred to as the common type of ChAT (cChAT), although alternative translation initiation generates two minor isoforms of 74- and 82-kDa with distinct N-terminal extension sequences (3–5). In addition, our group has previously reported an alternatively spliced variant of a rat ChAT homologue, which is predominantly localized in the peripheral nervous system and is therefore named peripheral ChAT (pChAT) (6).

ACh is synthesized in the neuronal cytoplasm including the nerve terminal, and then transported into synaptic vesicles by the vesicular acetylcholine transporter (VACHT). Upon neuronal depolarization, ACh is released into the synaptic cleft and binds to its specific receptors. Subsequently, intrasynaptic ACh is hydrolyzed by acetylcholinesterase to liberate choline and acetate. The liberated choline is immediately recycled into the presynaptic terminals by reuptake mainly mediated through the high-affinity choline transporter (CHT1). The rate-limiting step for ACh synthesis is the supply of choline (7, 8). Thus, the regulation of ACh synthesis in presynaptic neurons depends on VACHT and CHT1 as well as on ChAT (9). Despite the many studies carried out following the molecular cloning of these proteins (10–13), regulation of the expression of these coordinately functioning proteins remains to be clarified (14).

ChAT is also expressed in various types of non-neuronal cells including epithelial, mesothelial, endothelial, muscle, and immune cells (15). This observation suggests that ChAT is implicated in as yet unidentified function(s) besides neurotransmitter synthesis. Furthermore, Resendes et al. (17) have described nuclear localization of ChAT isoforms. This group has also defined one nuclear localization signal (NLS) in the 69-kDa isoform, and two NLSs in the 82-kDa isoform of human ChAT (hChAT) (16). An NLS acts like a “tag” on the exposed surface of a protein and targets it to the nucleus through the nuclear pore complex. The 69-kDa hChAT isoform is shuttled between the cytoplasmic and nuclear compartments, whereas the 82-kDa isoform is predominantly localized in the nucleus. Furthermore, we previously reported that a rat pChAT isoform, in contrast to the 69-kDa cChAT isoform, is preferentially localized in the cytoplasm and that inhibition of cellular protein kinase C activity leads to increased accumulation of nuclear pChAT (18). These observations led us to examine the functional relevance of the distinct subcellular localizations of ChAT.

To date the functional significance of nuclear ChAT remains unresolved. However, the 82-kDa nuclear isoform is expressed in human brains of normal aged subjects and in...
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ChAT Activity Assay—Cultured cells were washed twice with cold phosphate-buffered saline (PBS) (pH 7.4), and treated with 0.02% EDTA and 0.25% trypsin. The cells were pelleted by centrifugation at 80 \( \times \) g for 5 min and then homogenized in 5–10 volumes (v/w) of 10 mM phosphate buffer (PB) (pH 7.4) containing 0.5% Triton X-100. After centrifugation at 12,000 \( \times \) g for 20 min at 4 °C, 10 \( \mu \)l of the supernatant was used for ChAT activity measurement using the radiometric method with a slight modification (20). The reaction mixture consisted of 300 mM NaCl, 8 mM choline, 0.12 mM eserin, and 0.3 mM cold acetyl-CoA, and an appropriate quantity of \( [^{3}H] \) acetyl-CoA in 50 mM PB (pH 7.4). Each reaction was performed in a 0.6-ml plastic tube at 37 °C for 30 min. After stopping the reaction by ice cooling, the reaction mixture (20 \( \mu \)l total volume) was transferred to another tube containing 4.5 ml of 0.1 M PB (pH 7.4), followed by the addition of 2 ml of acetonitrile containing 5 mg/ml of sodium tetraphenylboron (Dojindo, Kumamoto, Japan) and 8 ml of a toluene-based scintillation mixture (Scintiblend II, Nacalai Tesque). The radioactivity of \( [^{3}H] \) ACh, collected by means of this liquid cation-exchange method into the organic phase,
was counted using a liquid scintillation counter (Packard Tri-CARB 3100, Meridian, CT). The ChAT activity was expressed as nanomoles of [3H]ACh formed per minute per milligram of protein (21). Protein concentrations were determined using the Bradford assay (Bio-Rad).

Confocal Laser Scanning Microscopy—Images were collected as previously reported (18). For comparison of the fluorescence intensity of individual images, identical parameters were set for imaging. Image processing and data analysis were performed using Photoshop or ImageJ software.

To evaluate transient nuclear translocation, we used the Crm-1-dependent nuclear export inhibitor, leptomycin B (Sigma) (22). Cultured cells were treated with 10 ng/ml of leptomycin B for up to 24 h. Analysis by confocal laser scanning microscopy was carried out as described above.

Immunocytochemistry for Cell Cycle Markers—We observed the relationship between the subcellular localization of ChAT-GFP and the cell cycle by immunocytochemistry for cell cycle markers. Immunocytochemistry was performed as previously reported (18). In brief, the cells were cultured for 2 days in a glass-bottom dish. After rinsing twice with cold PBS, the cells were fixed for 5 min with 4% paraformaldehyde in 100 mM phosphate buffer at 4 °C. The fixed cells were rinsed gently three times with 100 mM PBS containing 0.3% Triton X-100 (PBST). They were incubated overnight with anti-cyclin E antibody conjugated with rhodamine (rabbit polyclonal, Sc-481, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, 1:50) or anti-cyclin B1 antibody conjugated with Alexa 647 (mouse monoclonal, Santa Cruz Biotechnology, Inc., 1:50) in PBST at 4 °C. After washing 3 times with PBST for 10 min each, the cells were mounted with 4’,6-diamidino-2-phenylindole (DAPI)-Fluoromount-G (Southern Biotech, Birmingham, AL). Analysis by confocal laser scanning microscopy was carried out as described above.

Acetylcholine Antagonist and Agonists Treatment—To evaluate involvement of acetylcholine muscarinic receptors, cultured cells were treated with 10 nM to 10 μM atropine (Wako, Tokyo, Japan), a nonspecific muscarinic antagonist, for 72 h. We also tested the effects of nonspecific muscarinic agonists on CHT1 induction in the cultured cells, by using pilocarpine (10 μM to 1 μM, Wako) and carbachol (10 μM to 1 mM, Wako) for 72 h. Fresh aliquots of the agonist or antagonists were added to the culture at 24-h intervals with medium change. The treated cells were collected and analyzed using real time PCR as described below.

P38K Inhibitor Treatment—Cultured cells were treated with 1 to 50 μM LY294002 (Cayman Chemical, Ann Arbor, MI), a phosphatidylinositol 3-kinase (P38K) inhibitor, for 24 or 72 h (23). The treated cells were collected and analyzed using real time PCR as described below.

Real Time PCR—Real time reverse transcription PCR assays were performed to quantify the mRNA levels of CHT1, CHAT, and VACHT. Total RNA was extracted from cultured cells using the FastPure RNA kit (Takara-Bio, Otsu, Japan). Typically, 500 ng of total RNA was reverse-transcribed using the PrimeScript RT reagent kit (Takara-Bio) and random hexamers and oligo(dT) primers (Takara-Bio). To make external cDNA standards for real time PCR, the PCR products were subcloned into the pCR2.1 vector using a TA cloning kit (Invitrogen Japan Ltd., Tokyo, Japan). The plasmid cDNA was amplified in a One Shot TOP10 chemically competent Escherichia coli (Invitrogen Japan Ltd.), and isolated using a Quantum Prep Plasmid Miniprep kit (Bio-Rad Laboratories). The primers used for PCR of hChAT (CHAT), CHT1, VACHT, β-actin (ACTB), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown under supplemental Table S1.

Real time PCR was carried out using a Light Cycler system or the Light Cycler 480 instrument (Roche Diagnostics K.K., Tokyo, Japan) and the SYBR Premix Ex Taq (Perfect Real Time) II kit (Takara-Bio). To confirm amplification specificity, the PCR products from each primer pair were subjected to melting curve analysis and subsequent agarose gel electrophoresis. The results were analyzed using the second derivative maximum method of the LightCycler Software (Roche Diagnostics). Relative quantification of CDNA was performed based on these fluorescence measurements by comparison with a standard curve that was generated during the course of each PCR run. We confirmed a good correlation between the expression level of GAPDH mRNA and that of ACTB mRNA. These genes were therefore used as reference genes for normalization. The values were normalized to the levels of GAPDH cDNA determined from the same cDNA sample in the same PCR run. All experiments were performed independently at least three times. The levels of target mRNA were expressed relative to levels of GAPDH mRNA (arbitrary units).

Preparation of Cell Homogenates and Subcellular Fractions—For the preparation of total cell homogenates cultured cells were washed twice with cold PBS, and harvested using trypsin/EDTA. The cells were pelleted by centrifugation at 80 × g for 5 min and then lysed in 5–10 volumes (v/w) of 10 mM PB (pH 7.4) containing 0.5% Triton X-100 and a protease inhibitor mixture (Complete-mini, Roche Applied Science). The lysates were centrifuged at 12,000 × g for 20 min at 4 °C, and the supernatants were collected as total soluble fractions.

Nuclear fractions of cultured cells were obtained using a previously described method (24). Briefly, the SH-SY5Y cells were harvested in a buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.5 mM DTT) containing a protease inhibitor mixture. After rinsing the cells, 1/10 volume of 0.5% Nonidet-P 40 was added to the cell suspension. After the lysate was centrifuged at 1,000 × g for 2 min at 4 °C, the supernatant was collected as the cytoplasmic fraction. The resultant pellets were resuspended in a high-salt buffer (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 10% glycerol, 0.5 mM DTT) supplemented with protease inhibitors, and then placed on ice for 15 min. The supernatant was cleared by centrifugation at 12,000 × g for 15 min at 4 °C and collected as the nuclear fraction. CHT1 is functionally a plasma protein, which is also known to be constantly cycled through the plasma membrane and intracellular compartments. Because previous studies showed that CHT1 is predominantly intracellular, we used the cytoplasmic fractions for Western blotting of CHT1, as described below (25–29).
Western Blotting—Aliquots of cell lysates containing equal amounts of protein were subjected to Western blotting (18). The primary antibodies used were anti-GFP (mouse monoclonal, Roche, diluted 1:2500), anti-β-actin (mouse monoclonal, MAB1501, Chemicon International Inc., Billerica, MA, diluted 1:2000), anti-GAPDH (mouse monoclonal, MAB374, Chemicon, diluted 1:2000), anti-ChAT (rabbit polyclonal generated in our laboratory (30), diluted 1:5000), and anti-CHT1 (rabbit polyclonal, AB5966, Chemicon, diluted 1:1000). Bands were visualized using the Super-Signal West Pico chemiluminescent substrate (Thermo Fisher Scientific K.K, Yokohama, Japan), and analyzed using a LAS-4000 IR multicolor image analyzer (Fujifilm, Tokyo, Japan). The intensity of each band was semi-quantified using ImageJ software.

Statistical Analysis—The results are expressed as mean ± S.E. For statistical analysis, an unpaired two group t test, a non-parametric Kruskal-Wallis test, a Mann-Whitney test, or a post-hoc Tukey-Kramer test was used to determine the significance of differences between means.

RESULTS

Expression, Catalytic Activity, and Subcellular Localization of wt-hChAT-GFP—We first determined if the hChAT-GFP construct was useful for examination of ChAT activity and subcellular localization. The expected molecular weight of wt-hChAT fused with the 27-kDa GFP is 96 kDa. On immunoblotting of wt-hChAT-GFP-transfected HEK293 cell lysates, polyclonal anti-ChAT and monoclonal anti-GFP antibodies both detected wt-hChAT-GFP of the expected size (data not shown). A high level of ChAT-specific activity was detected in the homogenate of HEK293 cells transiently transfected with wt-hChAT-GFP. Furthermore, as we previously reported for a rat homolog of ChAT fused with GFP (18), wt-hChAT-GFP was observed in both the cytoplasm and nucleus in HEK293 cells by fluorescence microscopy (data not shown).

Characterization of Polyclonal SH-SY5Y Cell Lines Stably Expressing wt-hChAT-GFP—SH-SY5Y cells stably expressing wt-hChAT-GFP or GFP alone were selected based on the intensity of their GFP fluorescence. To avoid clonal deviation, we collected GFP-positive cells using FACS. The level of CHAT mRNA in each cell line was confirmed using RT-PCR. As expected, wt-hChAT-GFP cells expressed abundant CHAT mRNA, whereas GFP alone cells expressed only a trace level of CHAT mRNA (Fig. 2A).

Western blot analysis using polyclonal anti-ChAT or monoclonal anti-GFP antibodies confirmed the expression of a protein of the expected size in this cell line (Fig. 2B and data not shown). To ensure that the expressed ChAT-GFP was catalytically active, specific ChAT activities were measured. The wt-hChAT-GFP cells exhibited a high level of ChAT activity (wt-hChAT-GFP, 97.9 ± 7.16 nmol/mg of protein/min, mean ± S.E., and wt-hChAT-GFP-M, 12.6 ± 3.94), whereas no activity was detected in homogenates of GFP alone cells. ChAT activity of HEK293 cells stably expressing wt-hChAT-GFP was 158.68 ± 11.45 nmol/mg/min.

Subcellular distribution of wt-hChAT-GFP and GFP alone in these living cells was investigated using a confocal laser scanning microscope. As we previously reported for the rat ChAT-GFP homolog in HEK293 cells (18), wt-hChAT-GFP was located both in the cytoplasm and nucleus. Although it was more strongly expressed in the cytoplasm, wt-hChAT-GFP nuclear localization was assessed by its co-localization with the nuclear fluorescent dye Hoechst 33342 (Fig. 3A) and was confirmed using a biochemical cell fractionation assay (Fig. 3, B and C). The fluorescence intensity of GFP alone was the same in both the cytoplasm and nucleus, which may be a result of nonspecific passive diffusion.

We observed ChAT-GFP subcellular localization in wt-hChAT-GFP cells with cell-cycle markers: cyclin B1 (G2/M phase marker) and cyclin E (G1/S phase marker). In the cell culture condition we used in this study, strong nuclear staining with the anti-cyclin E antibody was detected in most of the tested wt-hChAT-GFP cells (supplemental Fig. S1), however, cyclin B1 staining was not detected.

Up-regulation of CHT1 mRNA Transcription in wt-hChAT-GFP Cells—To determine whether CHAT affects transcriptional activation of the CHT1 or VACHT genes, we analyzed RNA extracted from wt-hChAT-GFP and GFP alone cells for mRNA expression of these genes using real time PCR. The CHT1 mRNA level in wt-hChAT-GFP cells was significantly higher than that of GFP alone cells (Fig. 4B). VACHT mRNA in the wt-hChAT-GFP cells was comparable with that in GFP alone cells (relative ratio of wt-hChAT-GFP to GFP alone cells: 0.96 ± 0.02). These results suggested that transcription of CHT1 was selectively up-regulated by overexpression of wt-hChAT-GFP.

To examine whether the up-regulated CHT1 mRNA in the wt-hChAT-GFP cells was translated into protein, we performed Western blot analysis for CHT1. The anti-CHT1 antibody strongly detected CHT1-protein expression in the cytoplasmic fraction of wt-hChAT-GFP cells, whereas only a faint band was detected in GFP alone cell homogenates (Fig. 4C), suggesting that exogenous wt-hChAT-GFP induced CHT1 protein expression. In contrast, stable expression of wt-
hChAT-GFP (or of GFP alone) in HEK293 cells, which do not express CHT1 mRNA or protein, did not induce CHT1 mRNA or protein expression (data not shown), suggesting that CHT1 induction by wt-hChAT-GFP may be specific to neuronal cell types.

**CHT1 mRNA Induction Depends on the Nuclear Localization of hChAT**—To examine whether nuclear translocation of hChAT is required for its up-regulation of CHT1 expression, we prepared a mutant hChAT construct in which the NLS was disrupted. It has been reported that hChAT bears two putative NLSs: NLS1 (322RRLSEGDLFTQLRKIVKM339) and NLS2 (476ELPAPRRLRWK486) (16). We constructed two mutants: one of which was NLS1-(-)-hChAT-GFP, in which all four of the Arg322, Arg323, Arg334, and Lys335 residues of the NLS1 sequence were replaced by Ala, and the second was NLS2-(-)-hChAT-GFP, in which all four of the Arg481, Arg482, Arg484, and Lys486 residues of the NLS2 sequence were substituted by Ala (Fig. 1). Using fluorescence microscopy, we confirmed that mutation of NLS2 but not mutation of NLS1, inhibits hChAT-GFP translocation to the nucleus upon transient transfection into HEK293 cells (data not shown). This observation was consistent with a previous report that the C-terminal NLS2 is more important for hChAT nuclear translocation than NLS1 (16). We also confirmed that NLS2-(-)-hChAT-GFP exhibited ChAT activity equivalent to that of wt-hChAT-GFP in HEK293 cells (data not shown).

We next established a polyclonal SH-SY5Y cell line stably expressing NLS2-(-)-hChAT-GFP using a procedure similar to that used for establishment of wt-hChAT-GFP cell lines. We then compared the mRNA expression level of CHAT in these cells using real time PCR. The results indicated that equivalent levels of hChAT were expressed in wt-hChAT-GFP and NLS2-(-)-hChAT-GFP cells (Fig. 4A). In addition, NLS2-(-)-hChAT-GFP cells exhibited a significant level of CHAT activity (6.6 ± 1.05 nmol/mg/min). The ChAT activity of these cells was not as high as that of wt-hChAT-GFP cells, but was comparable with that of rat striatum homogenate (3.56 ± 0.12) (21).

Using confocal laser scanning microscopy, we determined that NLS2-(-)-hChAT-GFP fluorescence was almost exclusively localized in the cytoplasm, whereas nuclear fluorescence was similar to the background level (Fig. 3A). Co-staining with the Hoechst 33342 nuclear dye clearly showed that NLS2-(-)-hChAT-GFP did not co-localize with this dye. This cellular localization of NLS2-(-)-hChAT-GFP was in clear contrast to that of wt-hChAT-GFP. We further confirmed almost exclusive cytoplasmic localization of NLS2-(-)-hChAT-GFP using a biochemical cell fractionation assay. The isolated cytoplasmic and nuclear fractions from these cells were subjected to immunoblotting using antibodies against ChAT, GAPDH (as a cytoplasmic marker), or β-actin (as a loading control) (Fig. 3B). The nuclear fraction of NLS2-(-)-hChAT-GFP cells contained a very small amount of this.
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To exclude the possibility that the catalytic activity of NLS2-(−)-hChAT-GFP was not sufficient to induce CHT1 expression, we prepared another wt-hChAT-GFP cell with a moderate catalytic activity (wt-hChAT-GFP-M, 12.6 ± 2.27 nmol/mg/min), which was not significantly different from that of NLS2-(−)-hChAT-GFP (6.6 ± 1.05), and then compared CHT1 expression using quantitative RT-PCR. The CHT1 mRNA level in NLS2-(−)-hChAT-GFP cells was again significantly less than that of wt-hChAT-GFP cells. The CHT1 mRNA level in wt-hChAT-GFP-M cells was on the same level as wt-ChAT-GFP cells (Fig. 6).

ChAT Activity Is Necessary for Up-regulation of CH1 mRNA Expression—To address the question as to whether transcriptional activation of CHT1 by hChAT-GFP requires catalytic activity of ChAT or not, we prepared an enzymatically inactive ChAT-GFP construct harboring a E441K substitution, termed inactive-hChAT-GFP (Fig. 1). This Glu441 corresponds to one of the possible active sites (31). A E441K mutation in the CHAT gene is the cause of congenital myasthenic syndrome and results in the complete abolition of ChAT activity (1). We confirmed that no significant ChAT activity could be detected in homogenates of HEK293 cells transiently transfected with inactive hChAT-GFP.

We next established a polyclonal SH-SY5Y cell line stably expressing inactive hChAT-GFP using a method similar to that used for establishment of wt-hChAT-GFP cell lines. We then compared the expression levels of inactive hChAT-GFP in these cell lines using real time PCR (Fig. 4A). An equivalent level of CHAT mRNA was expressed in wt-hChAT-GFP and inactive hChAT-GFP cells. Using confocal microscopy and a biochemical subcellular fractionation assay, we further confirmed that the cytoplasmic and nuclear localization of inactive hChAT-GFP was similar to that of wt-hChAT-GFP (Fig. 3A). No ChAT activity was detected in homogenates of inactive hChAT-GFP cells.

Finally, we evaluated CHT1 expression in inactive hChAT-GFP cells. A real time PCR assay showed that the CHT1 mRNA level in these cells was equivalent to that in GFP alone.
cells (Fig. 4B). Western blotting of CHT1 protein levels revealed bands of approximately the same intensity in both inactive hChAT-GFP cells and GFP alone cells (Fig. 4C). These findings suggested that ChAT activity is indispensable for CHT1 induction by hChAT-GFP.

Muscarinic Antagonist Did Not Reverse the Up-regulation of CHT1 by ChAT—Because SH-SY5Y cells are known to be expressing muscarinic receptors on their cell surface (32, 33), we investigated whether the muscarinic receptors participate in the process of the observed CHT1 induction. Nonspecific muscarinic antagonist treatments did not effect the up-regulation of CHT1 by ChAT overexpression even with the highest concentration at 10 μM for 72 h (Fig. 7). Furthermore, the two tested agonists did not significantly change the level of CHT1 mRNA expression in either wt-hChAT-GFP cells or GFP alone cells (data not shown).

FIGURE 5. NLS2(−)-hChAT-GFP did not accumulate in the nucleus after leptomycin B treatment. The wt-hChAT-GFP (wt-hChAT) and NLS2(−)-hChAT-GFP (NLS2(−)) cells were treated with (+) or without (−) 10 ng/ml of leptomycin B (LMB), a potent inhibitor of nuclear export, for 8 h. GFP fluorescence and the fluorescence of the nuclear dye Hoechst 33342 were detected using a confocal laser scanning microscope. Bar = 20 μm. The relative intensity curves were made using ImageJ software.

FIGURE 6. Real time RT-PCR analysis of CHT1 mRNA expression in wt-hChAT-GFP-M and NLS2(−)-hChAT-GFP cells. Total RNA was extracted from wt-hChAT-GFP (wt-hChAT), wt-hChAT-GFP-M (wt-hChAT-M), NLS2(−)-hChAT-GFP (NLS2(−)), and GFP alone cells and was used as a template for a real time PCR assay. Values are the mean ± S.E. * indicates p < 0.05 significant difference by the non-parametric Mann-Whitney test, and ns represents not significant. Note that the catalytic activity of wt-hChAT-GFP-M cells was not significantly different from that of NLS2(−)-hChAT-GFP cells.

FIGURE 7. A muscarinic antagonist, atropine, did not reverse CHT1 induction by ChAT. Real time RT-PCR analysis of CHT1 in wt-hChAT-GFP cells treated with various concentrations of atropine for 72 h. Total RNA was then extracted from the cells and the relative level of CHT1 mRNA was evaluated by a real time PCR assay. Values are the mean ± S.E. No significant difference was detected by using a post hoc Tukey-Kramer test.
Inhibition of PI3K Reduced the Up-regulation of CHT1 by ChAT—A previous study showed that CHT1 expression is induced in primary septal cells by stimulation with nerve growth factor (NGF) (34). It has also been reported that treatment with an inhibitor of phosphatidylinositol 3-kinase (PI3K) prevents this effect of NGF on CHT1 expression (34). To determine whether PI3K inhibition could reverse CHT1 induction by ChAT, we treated wt-hChAT-GFP cells with 10 μM LY294002 in DMSO for 72 h. Although LY294002 treatment reduced the cell number of both wt-hChAT-GFP and GFP alone cell cultures, the mRNA expression level of CHAT mRNA in the surviving cells was not significantly different to that in cells treated with the same amount of control DMSO (Fig. 8A). The treatment of the wt-hChAT-GFP cells with LY294002 at 10 μM for 72 h induced a significant reduction in CHT1 mRNA expression (Fig. 8B). To determine whether PI3K inhibition could reverse CHT1 induction in a dose-responsive manner, we treated wt-hChAT-GFP cells with 1–50 μM LY294002 in DMSO for 24 h. We shortened the duration of treatment because at a concentration higher than 25 μM LY294002, the treated cells did not survive for 2 days. A significant correlation between the LY294002 concentration and reduction of the CHT1 mRNA level was observed (Fig. 8C).

**DISCUSSION**

Here we showed that overexpressed ChAT enhanced transcriptional activation of the CHT1 gene in human neuronal cell lines. This induction required nuclear translocation and the catalytic activity of ChAT. Our data are consistent with a previous paper by De Jaco et al. (35) who reported that an FB5 subclone of N18TG2 murine neuroblastoma cells in which a rat ChAT homologue was overexpressed showed a higher level of high affinity choline uptake compared with that in a ChAT-negative clone. Choline uptake, mediated by high affinity choline transporters, was assayed in that study rather than CHAT expression because CHT1 had not yet been cloned at the time of the study. To our knowledge, the present study is the first report demonstrating that nuclear ChAT acts as a transcriptional activator of selected target genes.

The underlying mechanism by which CHT1 gene expression is regulated remains unresolved. The CHT1 gene is located on chromosome 2; 2q12 (GenBank accession number NC_000002: region 107969427-107996876), whereas CHAT and VACHT are encoded by the cholinergic gene locus on chromosome 10; 10q11.2 (36). Many reports have demonstrated that the two genes encoding CHAT and VACHT are coordinately regulated by extracellular stimuli such as NGF, retinoic acids (RAs), and cytokines of the bone morphogenetic protein family (37–40). Synergic up-regulation of CHT1 and ChAT is also observed when superior cervical ganglia are treated with leukemia inhibitory factor (41). It has been shown that simultaneous up-regulation of CHT1 and VACHT is induced when primary septal cells are stimulated with NGF (34). The effects of NGF on cholinergic gene expression are known to be dependent on PI3K signaling (42), and treatment with an inhibitor of PI3K inhibits the effect of NGF on CHT1 expression (34). Madziar et al. (43) also revealed that NGF regulates the expression of the cholinergic locus and CHT1 via the Akt/protein kinase B signaling pathway in pheochromocytoma 12 cells and mouse primary septal cells. On the other hand, it has been reported that regulation of CHT1 expression is independent of the cholinergic gene locus under some conditions. Treatment of superior cervical ganglia neurons with RA up-regulated ChAT expression but down-regulated CHT1 mRNA expression (41). Berse et al. (34) also reported that all-trans-RA treatment increased VACHT expression in cultured mouse septal neurons, but that CHT1
expression was unaffected. Brock et al. (44) showed a differential regulation of CH1 and the cholinergic locus by cAMP signaling pathways in NSC-19 cells. Up-regulated CH1 expression in vivo has been reported to compensate for cholinergic dysfunction due to ChAT haploinsufficiency in mutant mice (45). In the present report, we showed that ChAT itself could induce the expression of CH1, suggesting that ChAT might act in a positive feedback loop to maintain cholinergic phenotypes during neuronal differentiation. Our results further suggest that activation of CH1 transcription by ChAT depends on PI3K signaling. The reduction of ChAT-induced CH1 transcription was significantly correlated with the concentration of LY294002 used for the treatment. A previous report showed that LY294002 treatment reduces endogenous CH1 expression in mouse primary septal culture as well as NGF-induced CH1 expression (34). Our observation of reduction in CH1 expression by LY294002 appears to be consistent with the LY294002 effect on endogenous CH1 expression. The expression of CH1 in non-neuronal tissues has also been reported (46–51). However, because we did not detect this positive feedback loop in non-neuronal HEK293 cells, the regulation of CH1 expression might differ between neuronal and non-neuronal cells. We speculated that one of the following two potential molecular mechanisms might underlie CH1 induction by ChAT: 1) ChAT enhances the production of ACh, which acts on ACh receptors on the cell surface via an autocrine loop and results in induction of CH1 expression, or 2) nuclear ChAT directly activates the transcriptional machinery important for CH1 gene transcription.

Our result that enzymatically active but exclusively cytoplasmic NLS2-(−)–hChAT could not induce CH1 expression indicated that the former mechanism may be implausible. The fact that wt-ChAT-GFP-M cells, which have the same level of enzymatic activity as NLS2-(−)–hChAT, induced CH1 mRNA expression indicates that NLS2-(−)–hChAT may satisfy the conditions for the induction of CH1 in terms of enzymatic activity. In the opposite situation, inactive nuclear hChAT also could not enhance CH1 transcription at all. These results suggested that nuclear ChAT activity is required for up-regulation of CH1 transcription, although we cannot exclude the possibility that Glu441 is critical for both ChAT activity and CH1 transcriptional induction.

SH-SY5Y cells are reported to be expressing muscarinic receptors (32, 33). To clarify whether ACh receptors on the cell surface did not participate in the CH1 mRNA induction by ChAT overexpression, we performed the pharmacologic study using a muscarinic antagonist and muscarinic agonists. Atropine up to 10 μM could not reverse the up-regulation of CH1 expression by ChAT, and either pilocarpine or carbachol up to 1 mM did not enhance the induction of CH1. These results indicate that cell surface ACh receptors do not modulate this induction.

If this is the case, then, how is it possible that nuclear ChAT activates CH1 transcription? One possible mechanism is that ACh generated by ChAT acts via acetylcholine receptors existing inside of the cells. G-protein-coupled muscarinic ACh receptors have been detected in the nuclear enve-
