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Cell Type-specific Regulation of Choline Acetyltransferase Gene Expression

ROLE OF THE NEURON-RESTRICTIVE SILENCER ELEMENT AND CHOLINERGIC-SPECIFIC ENHANCER SEQUENCES*

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This study demonstrates the presence of positive and negative regulatory elements within a 2336-base pair-long region of the rat choline acetyltransferase (ChAT) gene promoter that cooperate to direct cell type-specific expression in cholinergic cells. A 21-base pair-long neuron-restrictive silencer element (NRSE) was identified in the proximal part of this region. This element was recognized by the neuron-restrictive silencer factor (NRSF), previously shown to regulate expression of other neuron-specific genes. The ChAT NRSE was inactive in both cholinergic and non-cholinergic neuronal cells, but repressed expression from a heterologous promoter in non-neuronal cells. Specific deletion of this element allowed ChAT gene promoter activity in non-neuronal cells, and overexpression of NRSF repressed ChAT gene promoter activity in cholinergic cells. The distal part of the ChAT gene promoter showed cholinergic-specific enhancing activity, which stimulated promoter activity in cholinergic cells, but was inactive in non-cholinergic neuronal and non-neuronal cells. This enhancer region suppressed the activity of the ChAT NRSE in cholinergic cells, even after NRSF overexpression. Thus, at least two kinds of regulatory elements cooperate to direct ChAT gene expression to cholinergic neurons, namely a neuron-restrictive silencer element and a cholinergic-specific enhancer.

A fully developed nervous system is characterized by a complex organization of interconnected neurons, each expressing a specific complement of neurotransmitters and neuromodulators. The elucidation of the molecular mechanisms that govern the acquisition and plasticity of the neurotransmitter phenotype of different neuronal subpopulations remains a major challenge in neurobiology. Choline acetyltransferase (ChAT)[1] (EC 2.3.1.6) is the rate-limiting enzyme in the synthesis of acetylcholine, one of the main neurotransmitters of the nervous system. Cholinergic neurons in the central nervous system include cells in the basal forebrain, cortex, motor nuclei, and spinal cord. These neurons acquire their cholinergic phenotype progressively as the expression of the ChAT gene increases during development. In motor neurons of the spinal cord, the levels of ChAT mRNA increase slowly during embryonic life and more rapidly after birth, reaching maximal levels in the adult (1). Developing neurons may switch from a non-cholinergic to a cholinergic phenotype only after reaching the target of innervation (2) and may also become cholinergic in response to injury (3). In most of these circumstances, a major point of control of the cholinergic phenotype has been demonstrated at the transcriptional level (4, 5), suggesting the presence of regulatory elements in the ChAT gene controlling accurate time- and cell-specific expression.

The ChAT gene comprises several exons including a number of alternatively spliced 5′-noncoding exons (6). Transcription of the rat and mouse ChAT genes appears to initiate at multiple promoters, resulting in the production of five different 5′-mRNA sequences (6–9). Different upstream regions of the ChAT gene have been shown to be necessary for cell type-specific expression of reporter genes in cholinergic cell lines (6, 10–14). We have previously shown that a 3862-bp-long, but not a 1520-bp-long, 5′-flanking segment from the rat ChAT gene promoter directs cell type-specific expression of a reporter gene in cholinergic cells *in vitro* (10), suggesting that the intervening 2336-bp-long segment contains elements responsible for the cell type-specific expression of the ChAT gene. In support of this notion, we have recently demonstrated that this segment from the ChAT gene promoter is able to confer cholinergic specificity to a non-cholinergic promoter in an orientation-independent manner and to direct correct developmental and tissue-specific expression to cholinergic cells in transgenic mice (15). We now report the functional dissection of this region and the identification of a neuron-restrictive silencer element and cholinergic-specific enhancer sequences that cooperate to direct cell type-specific expression in cholinergic neurons.

MATERIALS AND METHODS

Plasmid Construction and Site-directed Mutagenesis—A 2336-bp *Eco*RI-*Hin*dIII fragment from a rat ChAT genomic λEMBL3 clone (10) was isolated and subcloned into pBSKS$^+$ (Stratagene, La Jolla, CA). Nested deletions (Nested Deletion kit, Pharmacia, Uppsala) were made from both 3′- and 5′-ends of the fragment and confirmed by DNA sequence analysis. Heterologous promoter constructs were made by subcloning fragments into the *Sal*I site of pBLCAT$_2$ (16). Deletion of the ChAT NRSE was made by oligonucleotide site-directed mutagenesis as described by Kunkel (17) using the oligonucleotide 5′-TAAACACCG-GCTCCTAGGAACTGGGGAGCCGCTCTACCTCCTGGC-3′ and was confirmed by DNA sequence analysis.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) X92751.

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[1] The abbreviations used are: ChAT, choline acetyltransferase; bp, base pair(s); NRSE, neuron-restrictive silencer element; NRSF, neuron-restrictive silencer factor; CAT, chloramphenicol acetyltransferase; tk, herpes simplex virus thymidine kinase; BDNF, brain-derived neurotrophic factor; RPA, ribonuclease protection assay; REST, RE1-silencing transcription factor.

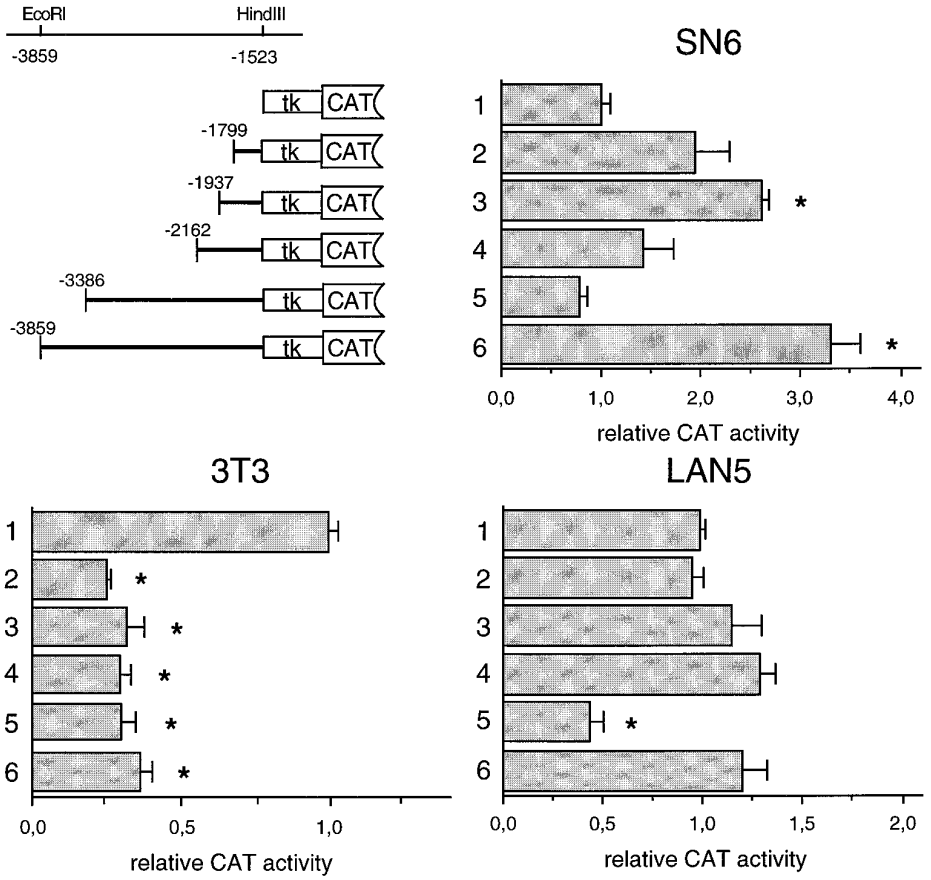

FIG. 1. **5′-Deletion analysis of the upstream 2336-bp *Eco*RI-*Hin*dIII regulatory region of the rat ChAT gene linked to a heterologous promoter.** Heterologous promoter constructs containing the tk promoter were transiently transfected into the mouse cholinergic neuronal SN6, human non-cholinergic neuronal LAN5, and rat FR3T3 fibroblast cell lines. Cells were harvested after 48 h, lysed, and subjected to CAT reporter gene activity assay. A simplified map of the corresponding region of the ChAT genomic clone is shown. The 156-bp sequence containing the tk promoter is not drawn to scale. *Bars* show mean ± S.E. *, $p < 0.001$ *versus* the tk construct (Student's *t* test).

RNA Preparation and RNase Protection Assay—Total RNA was prepared from frozen tissue by the guanidine isothiocyanate/CsCl method (18). Polyadenylated RNA was purified by chromatography on an oligo(dT)-cellulose column. Total RNA from cell lines was purified by the method described by Chomczynski and Sacci (19). The recovery of RNA was quantified spectrophotometrically. RNase protection assays were performed using the RPAII Ribonuclease Protection Assay kit (Ambion Inc., Austin, TX). The cRNA probes were a 450-bp *Pvu*II-*Pst*I fragment from mouse NRSF, a 401-bp fragment spanning positions 640-1140 of rat NRSF, and a 426-bp *Pvu*II-*Pst*I fragment from human NRSF (20) cloned into pBSKS$^+$. For determination of cell line mRNA levels, standard curves were made with spectrophotometrically quantified sense cRNA produced from the same plasmids. Probes were labeled with [α-^{32}P]CTP by run-off transcription from the linearized plasmid. 2.5×10^5 cpm of probe and 10 μg of RNA were used in each hybridization. The protected fragments were separated on a 5% denaturing polyacrylamide gel and subsequently exposed to x-ray film.

Cell Culture and Transfections—Fischer rat fibroblast (FR3T3), mouse neuroblastoma (N2A), mouse basal forebrain cholinergic neuron (SN6), mouse motor neuron (2F1.10.14, referred to here as MN1), and human neuroblastoma (LAN5) cell lines were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum supplemented with 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Life Technologies, Inc.) at 37 °C in a 5% CO_2 atmosphere. SN6 is a cholinergic neuronal cell line derived from a fusion of mouse primary septal neurons with the mouse neuroblastoma cell line N18TG2 (21). MN1 is a cholinergic motor neuron cell line derived from a fusion of N18TG2 with embryonic mouse spinal cord motor neurons (clone 2F1.10.14.7) (22). Cells grown to ~70% confluency were transfected according to the calcium phosphate procedure. 35-mm wells were transfected with 1.5 μg of DNA plus 0.5 μg of the reference plasmid pON260 (23) expressing β-galactosidase as an internal control for transfection efficiency. For cotransfections, 1 μg of reporter plasmid and 4 μg of NRSF-expressing pCMV-HZ4 (20) or parental vector pcDNA3 (Invitrogen) were used. Cells were harvested after 48 h in culture with phosphate-buffered saline containing 10 mM EDTA, transferred to 250 mM Tris (pH 7.5), and lysed by five cycles of freeze-thawing. Samples were equalized in protein content (24) and were incubated at 65 °C for 5 min to destroy endogenous acetyltransferases and acetylases. CAT assays were performed as described previously (25), and percentage conversion was calculated after scintillation counting of TLC plates. The conversion after a 2-h reaction from transfections of the construct with the tk promoter alone was 5, 10, and 9% for the FR3T3, SN6, and LAN5 cell lines, corresponding to 9, 17, and 15 nmol of converted acetyl-coenzyme A, respectively.

Electrophoretic Mobility Shift Assay—Human NRSF was produced by *in vitro* translation from the pREST-H1 plasmid (20) using the TNT Coupled Reticulocyte Lysate system (Promega, Madison, WI). The product was analyzed by SDS-polyacrylamide gel electrophoresis. Nuclear extracts were made according to Andrews and Faller (26). Binding reactions were performed in 20 μl of 20 mM HEPES (pH 7.6), 0.1% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol, 2.5 mM $MgCl_2$, 250 mM KCl, and 2 μg of poly(dI-dC)·poly(dI-dC). 5 μl of *in vitro* translation product or 10 μg of nuclear extract was preincubated in buffer on ice for 10 min; 1×10^4 cpm of probe and competitor were added; and the reaction was incubated at room temperature for 20 min. For supershift experiments, 1 μl of either polyclonal antiserum raised against the zinc-finger region of mouse NRSF (20) or preimmune serum was then added, and the reaction was incubated for another 30 min at room temperature. The binding reaction was loaded onto a 6% nondenaturing polyacrylamide gel and run in 0.25 × Tris borate/ETDA buffer. The ChAT NRSE oligonucleotide probe was labeled with [α-^{32}P]dCTP by Klenow fill-in. Oligonucleotides used were as follows: ChAT NRSE, TAGGAACTGTCCAGCACCACGGACAGTTCCGGGAGCCGC; SCG10 NRSE, AGCTGCAAAGCCATTTCAGCACCACGGAGAGTGCCTCTGC; and unrelated control, CCGTTGATATATCCCAATGGC.

RESULTS

Analysis of Regulatory Regions Directing Cell Type-specific Expression of the ChAT Gene Promoter—We previously isolated a distal region from the ChAT gene promoter containing a cell type-specific silencer-like activity that restricts ChAT gene expression to cholinergic cells *in vitro* (10). This region encompasses 2336 bp of 5′-flanking sequence and extends from an *Eco*RI site at position −3859 to a *Hin*dIII site at position −1523. (The transcriptional start site, corresponding to position 498 of the sequence published in Ref. 10, is herein defined

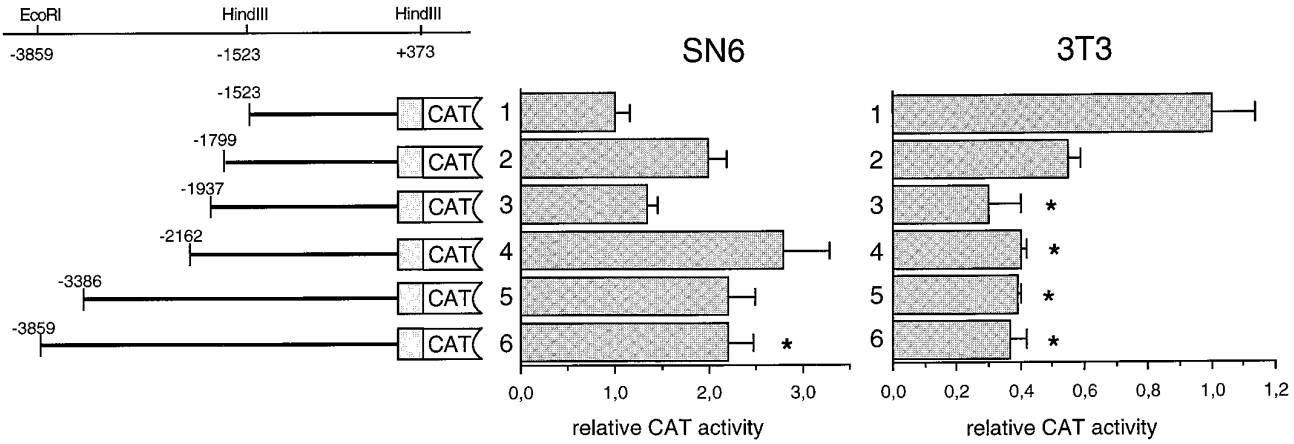

FIG. 2. **5′-Deletion analysis of the upstream 2336-bp *Eco*RI-*Hin*dIII regulatory region of the rat ChAT gene.** Homologous promoter constructs were transiently transfected into the mouse cholinergic neuronal SN6 and rat 3T3 fibroblast cell lines. Cells were harvested after 48 h, lysed, and subjected to CAT reporter gene activity assay. A simplified map of the corresponding region of the ChAT genomic clone is shown. *Shaded boxes*, ChAT noncoding first exon sequence. *Bars* show mean ± S.E. *, $p < 0.05$ *versus* the −1523 construct (Student's *t* test).

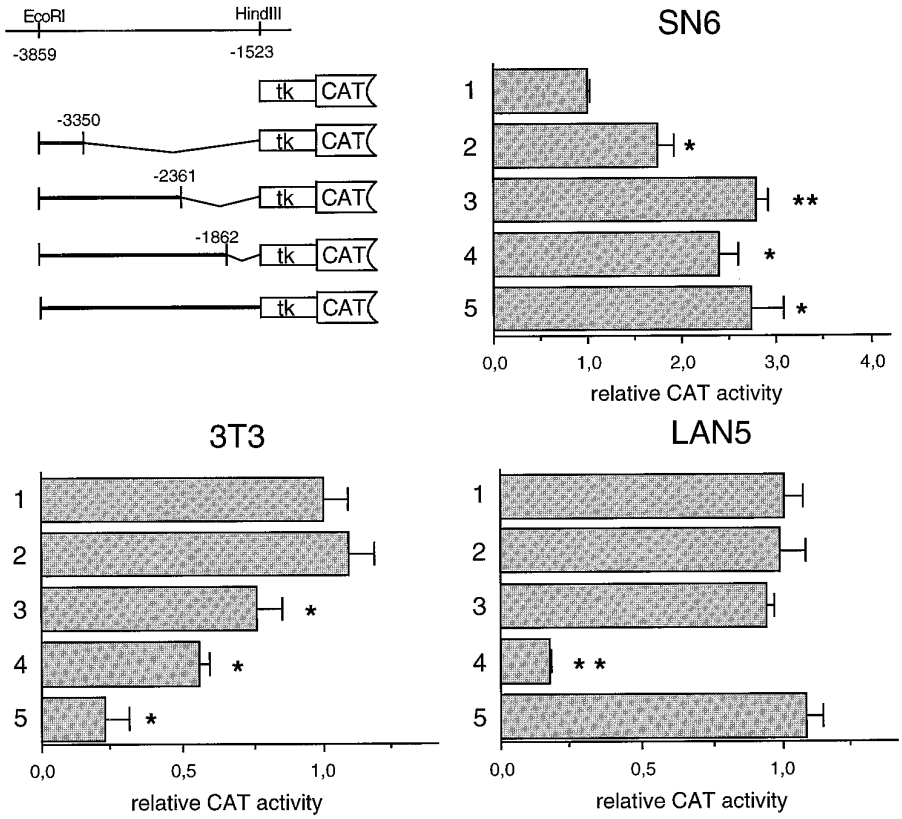

FIG. 3. **3′-Deletion analysis of the upstream 2336-bp *Eco*RI-*Hin*dIII regulatory region of the rat ChAT gene.** Heterologous promoter constructs containing the tk promoter were transiently transfected into the indicated cell lines. Cells were harvested after 48 h, lysed, and subjected to CAT reporter gene activity assay. A simplified map of the corresponding region of the ChAT genomic clone is shown. The 156-bp sequence containing the tk promoter is not drawn to scale. *Bars* show mean ± S.E. *, $p < 0.05$; **, $p < 0.001$ *versus* the tk construct (Student's *t* test).

as +1.[2]) This region was recently shown to be able to direct cholinergic-specific expression of a tk gene promoter *in vitro* and in transgenic mice (15). To further characterize sequences within this region controlling cell-type specificity, a series of 5′-deletions were generated between positions −3859 (*Eco*RI) and −1523 (*Hin*dIII) of the ChAT gene and subcloned upstream of a tk gene promoter linked to the bacterial CAT reporter gene. The fragment of the tk promoter used allows robust transcription in a variety of neuronal and non-neuronal cell lines; a plasmid construct without promoter has no detectable activity. In the cholinergic neuronal SN6 cell line, this analysis revealed a region with moderate enhancing activity between positions −1523 and −2162 and another enhancer upstream of position −3386 (Fig. 1). In agreement with our previous results (15), the presence of the 2336-bp segment from the ChAT gene (−3859 construct) suppressed the activity of the downstream tk promoter in the fibroblast cell line FR3T3 (Fig. 1). Progressive deletions from the 5′-end of this region did not show major differences in the transcriptional efficiency of promoter constructs in FR3T3 cells (Fig. 1), indicating the presence of a silencing activity in the 3′-end of this region. The constructs were also analyzed in LAN5, a non-cholinergic human neuroblastoma cell line. In LAN5 cells, none of the 5′-deletion constructs showed a significant influence of the tk promoter activity, with the exception of the −3386 construct, which showed a reduction in activity similar to that seen in SN6 cells (Fig. 1). Similar results were obtained in an equivalent series of 5′-deletions performed in a native ChAT gene

[2] P. Lönnerberg and C. F. Ibáñez, unpublished data.

promoter construct (intact 5′-region with its homologous promoter) (Fig. 2).

Regulatory elements in the 2336-bp *Eco*RI-*Hin*dIII fragment were further investigated in a series of 3′-deletions. Deletions upstream of position −2361 reduced the activity of the construct in SN6 cells, consistent with the presence of a region with enhancing activity in cholinergic cells (Fig. 3). In contrast, these deletions progressively increased promoter activity in FR3T3 cells (Fig. 3). Deletion of the most downstream 339 bp resulted in a 3-fold increase in transcription in FR3T3 fibroblast cells (Fig. 3). A further 2-fold increase was obtained after 3′-deletion of an additional 499 bp, indicating another region with suppressing activity in FR3T3 cells. Promoter activity was not substantially influenced by the 3′-deletions in LAN5 cells, with the exception of the −1862 construct, which suppressed tk promoter activity (Fig. 3). Taken together, data from deletion analyses indicated the presence of a silencing activity in non-neuronal cells between positions −1862 and −1523 and several upstream segments that cooperated to give enhancing activity in cholinergic cells.

rat ChAT	TCCAGCACCACGGACAGTTCC
rat SCG10	.T...C.....G...G..
rat NaII	.T...A.....CA..
human synI	.T.....G.....G..
rat BDNF	.T.....TT.....AG..
human DBH	AG....T..T.....CTCA-G
rat α3NKA	CTT...TT.T...TGG-CG..

FIG. 4. **Alignment of NRSE core sequences from different neuron-specific genes.** Shown are NRSEs from the ChAT, SCG10 (27), type II sodium channel (*NaII*) (29), synapsin I (*synI*) (28), BDNF (31), dopamine β-hydroxylase (*DBH*) (30), and Na,K-ATPase α_3-subunit (*α3NKA*) (33) genes.

Localization of a NRSE in the ChAT Gene Promoter—4000 bp of the upstream region of the ChAT gene promoter were sequenced and scanned for the presence of previously described regulatory sequences (this sequence is available from the EMBL Nucleotide Sequence Data Bank under accession number X92751). This search resulted in the identification of a 21-bp sequence (from positions −1580 to −1560) highly related to the consensus sequence of the NRSE described previously in other neuron-specific genes (Fig. 4) (27). This element has been shown to repress expression in non-neuronal cells of several neuronal genes including rat SCG10 (27), human synapsin I (28), and rat type II sodium channel (29) genes, and it has also been found in the promoter regions of several other neuron-specific genes such as human dopamine β-hydroxylase (30) and rat brain-derived neurotrophic factor (BDNF) (31). We therefore investigated the functional role of the ChAT NRSE-like element in determining cell-type specificity in the ChAT gene promoter. For this purpose, the 21-bp element was deleted by site-directed mutagenesis in two heterologous ChAT gene promoter constructs, *i.e.* positions −3859 to −1523 and positions −1799 to −1523, containing the tk promoter and the CAT reporter gene. The activity of these constructs was compared with that of the wild-type sequences and with the activity of the tk promoter alone. The deletion did not affect the activity of the −3859 and −1799 constructs in cholinergic SN6 cells as assessed by CAT activity (Fig. 5). In FR3T3 fibroblast cells, deletion of the NRSE-like element increased promoter activity, consistent with the role of the NRSE as a non-neuronal silencer (Fig. 5). In the short construct (−1799), the deletion restored

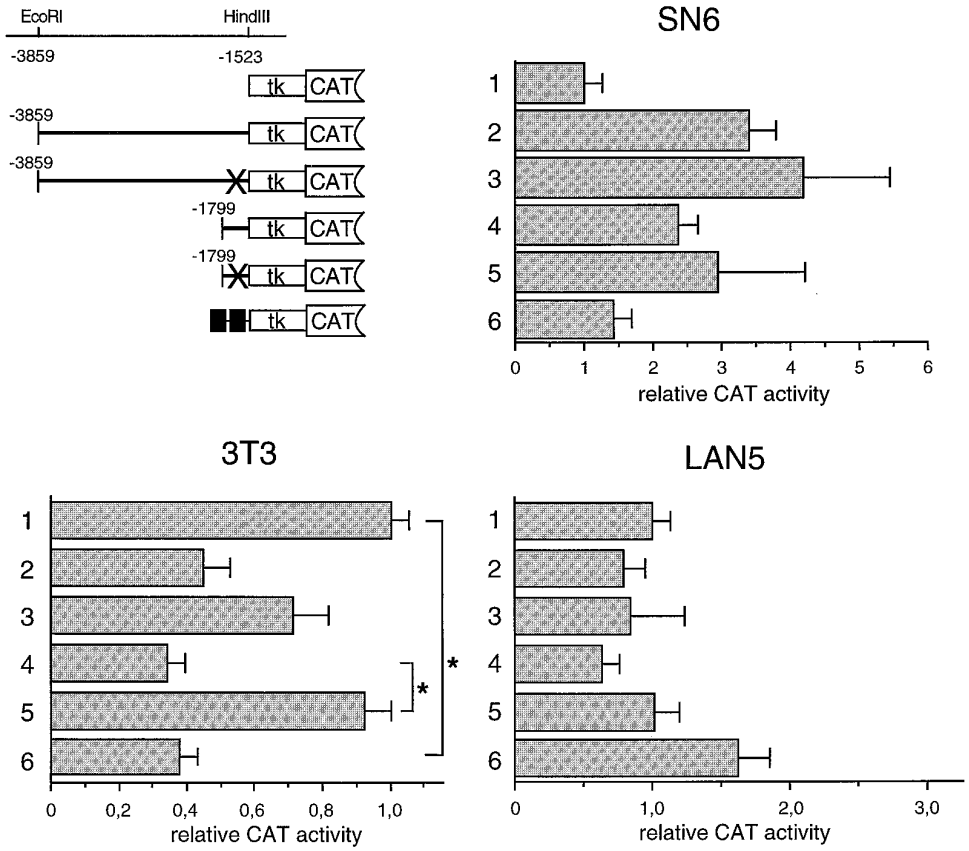

FIG. 5. **Role of the ChAT NRSE in determining cell-specific gene expression.** The 21-bp sequence at position −1579 containing the ChAT NRSE was deleted by site-directed mutagenesis in heterologous tk promoter constructs containing ChAT upstream regulatory sequences. After transient transfection into the indicated cell lines, CAT reporter gene activity was assayed. A simplified map of the corresponding region of the ChAT genomic clone is shown. *X* denotes the deleted NRSE. *Solid boxes* represent two tandem copies of a 39-bp oligonucleotide spanning the ChAT NRSE. *Bars* show mean ± S.E. ($n = 3$). *, $p < 0.005$ *versus* the tk construct (Student's *t* test).

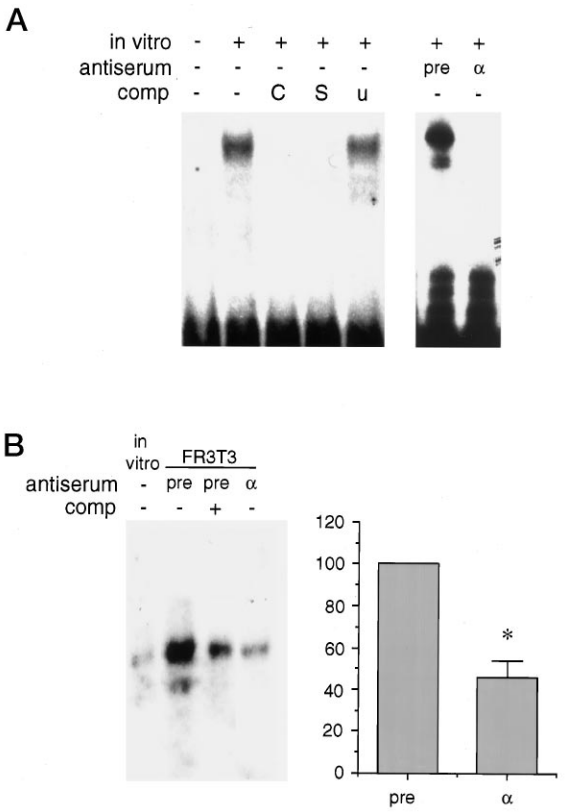

FIG. 6. **Binding of NRSF to the ChAT NRSE.** *A*, an N-terminal fragment of human NRSF containing the zinc-finger domains was produced by *in vitro* translation in a rabbit reticulocyte lysate. Electrophoretic mobility shift assays were performed with one-tenth of the *in vitro* translation product (*in vitro*) and a ^{32}P-labeled 39-bp oligonucleotide spanning the rat ChAT NRSE as probe, with or without the addition of competitor oligonucleotides (*comp*) at a 500-fold molar excess. Competitors used were the unlabeled probe oligonucleotide (control (*C*)), the NRSE from the rat SCG10 gene (*S*), and an unrelated oligonucleotide derived from the pBLCAT$_2$ plasmid sequence (*u*). A polyclonal antiserum (α) raised against a recombinantly produced NRSF fragment containing the zinc-finger domain was used to block binding of NRSF to the probe. *pre*, preimmune serum. *B*, 10 μg of FR3T3 nuclear protein extracts was used in electrophoretic mobility shift assay with the ChAT NRSE oligonucleotide probe. Note that the unlabeled oligonucleotide (*comp*) and anti-NRSF antibodies (α) compete binding to the probe. Full-length human NRSF produced by *in vitro* translation in a rabbit reticulocyte lysate was included for identification of the NRSF-derived band (*in vitro lane*). The histogram shows the quantitation of three independent experiments comparing preimmune and anti-NRSF antisera. Optical density values with preimmune serum were arbitrarily set to 100. *Bars* show mean ± S.E. *, $p < 0.01$ (one-group *t* test; $n = 3$).

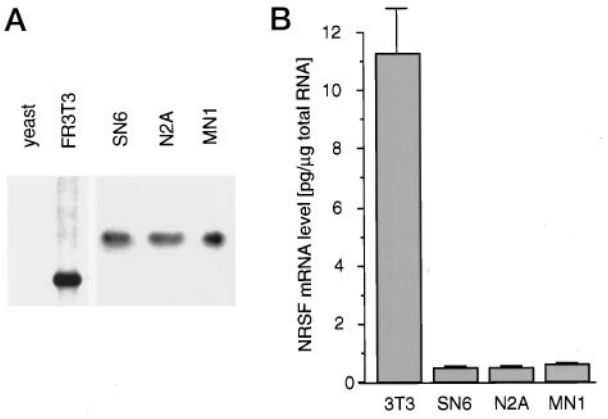

FIG. 7. **Analysis of NRSF mRNA expression in different cell lines.** *A*, 10 μg of total RNA from the indicated cell lines was used in a RPA with cRNA probes derived from a 450-bp fragment of a mouse NRSF cDNA or a 401-bp fragment of a rat NRSF cDNA. Yeast tRNA was used as a negative control. *B*, shown is the quantitation of the RPA analysis of NRSF mRNA. *Bars* show picograms of mRNA/10 μg of total RNA and represent mean ± S.E. ($n = 2$).

the level of CAT activity to that of the tk promoter alone (Fig. 5). Deletion of the ChAT NRSE had no major effect in LAN5 cells. Further evidence of the ability of the ChAT NRSE to control neuron-specific gene expression was obtained by introducing a tandem NRSE oligonucleotide (2×NRSE) upstream of the tk promoter and the CAT reporter gene. Isolated from its native sequence context, the tandem NRSE repressed the activity of the tk promoter by ~3-fold in FR3T3 cells, but not in SN6 or LAN5 cells (Fig. 5). Combined with the data from the serial deletion analysis, these results suggest that the ChAT NRSE contributes to the neuron-specific expression of the ChAT gene promoter.

Binding of the Neuron-restrictive Silencing Factor to the ChAT NRSE—A DNA-binding protein has been cloned on the basis of its ability to bind to NRSEs in the SCG10 and type II sodium channel genes (20, 32). We investigated whether this protein, NRSF (also called REST), was able to interact with the NRSE of the ChAT gene. Electrophoretic mobility shift assays were performed with human NRSF *in vitro* translated from a construct containing the zinc-finger DNA-binding domain (20) and a 39-bp oligonucleotide probe containing the ChAT NRSE and nine nucleotides each of 5′- and 3′-flanking sequence. Human NRSF bound specifically to the ChAT NRSE in this assay (Fig. 6*A*). Binding was prevented by excess nonradioactive ChAT NRSE oligonucleotide or by an oligonucleotide containing the NRSE from the SCG10 gene (Fig. 6*A*). An oligonucleotide with a sequence unrelated to NRSEs did not compete NRSF binding (Fig. 6*A*). However, binding was prevented by a polyclonal antibody directed against a recombinantly produced N-terminal fragment of NRSF containing the zinc-finger domain (20). These data show that NRSF is able to specifically bind to the ChAT gene NRSE.

Next, we investigated whether FR3T3 cells contained NRSE binding activity. Electrophoretic mobility shift assay performed with FR3T3 nuclear extract revealed complexes of the expected mobility (Fig. 6*B*). Binding was competed by an excess of unlabeled oligonucleotide and by the polyclonal antiserum raised against the zinc-finger domain of mouse NRSF (Fig. 6*B*). In three independent experiments, anti-NRSF antibodies caused a 60% reduction in binding compared with preimmune serum (Fig. 6*B*). Because the anti-NRSF antiserum had been raised against the N-terminal fragment of NRSF, the incomplete inhibition might have been due to C-terminal regions in native NRSF partially blocking access of the antibody. Combined, these data demonstrate the presence of a NRSE binding activity in FR3T3 cells that is immunologically related to NRSF.

Expression of NRSF mRNA in Different Cell Lines—These results prompted us to investigate the endogenous expression of NRSF in different cholinergic and non-cholinergic cell lines. Ribonuclease protection assays (RPAs) were performed with equal amounts of total RNA extracted from various cell lines using riboprobes derived from the mouse and rat NRSF/REST genes (20, 32). In addition to SN6 and FR3T3 cells, a mouse cholinergic motor neuron hybrid cell line (MN1) (22) and a mouse neuroblastoma cell line (N2A) were also analyzed and compared (Fig. 7*A*). mRNA was quantitated against standard curves made by RPA of various amounts of sense cRNA (data not shown). NRSF mRNA levels in FR3T3 cells were ~30-fold higher than in neuronal cell lines (Fig. 7*B*). Similarly, using a

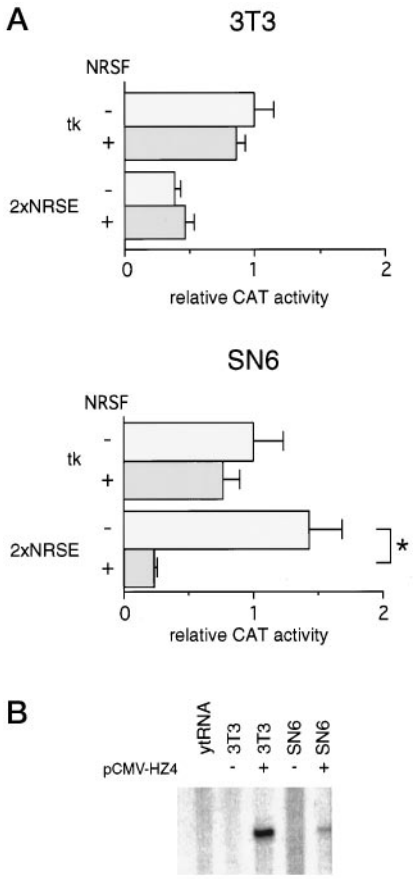

FIG. 8. **Effect of NRSF overexpression on the activity of a ChAT NRSE-linked tk promoter.** Reporter gene constructs were made containing either a tk promoter alone (*tk*) or two tandem copies of the rat ChAT NRSE in the sense orientation in front of the tk promoter (*2×NRSE*) in front of the chloramphenicol acetyltransferase reporter gene. The expression plasmid pCMV-HZ4, producing human NRSF (+), or a control plasmid (−) was transfected at a 4-fold molar excess with respect to reporter constructs, and 2 days after transfection, cell lysates were analyzed. *A*, reporter gene activity assays. The mean activity of the tk promoter alone was arbitrarily set to 1. *Bars* show mean ± S.E. ($n = 3$). *, $p < 0.025$ (Student's *t* test). *B*, ribonuclease protection assay analysis of human NRSF mRNA expression in cell lines cotransfected with pCMV-HZ4 and the 2×NRSE construct. 10 μg of total RNA was analyzed in each reaction. Yeast transfer RNA (*ytRNA*) was used as a negative control. Note that the human NRSF riboprobe does not detect endogenous NRSF mRNA in these cell lines.

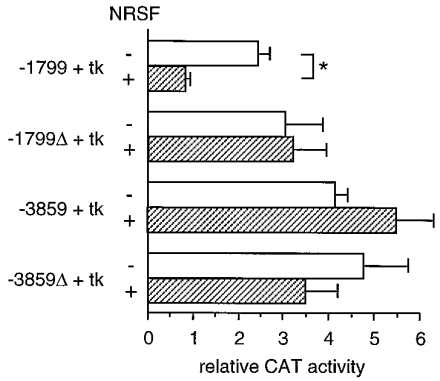

FIG. 9. **Effects of NRSF overexpression on the activity of regulatory sequences from the ChAT gene in SN6 cells.** CAT reporter gene plasmids containing the complete 2336-bp regulatory fragment from the ChAT gene (*−3859 + tk*) or only the most proximal 276 bp from this fragment (*−1799 + tk*) in front of a tk promoter were transiently cotransfected with a 4-fold molar excess of either a human NRSF expression plasmid (*hatched bars*) or a control plasmid (*open bars*). For comparison, constructs with a deletion in the ChAT NRSE (*−3859Δ + tk* and *−1799Δ + tk*, respectively) were also included. 48 h after transfection, cells were harvested, and CAT reporter gene activity in cell lysates was measured. The activity of the tk promoter alone was arbitrarily set to 1. *Bars* show mean ± S.E. ($n = 3$). *, $p < 0.05$ (Student's *t* test).

human NRSF riboprobe, low levels of NRSF mRNA were detected in LAN5 cells, but 10-fold higher levels were seen in the non-neuronal cell line HeLa (data not shown). In agreement with these data, low levels of NRSE binding activity were found in nuclear extracts of SN6 cells (data not shown).

Silencing Activities of Overexpressed NRSF in Cholinergic Cells—Previous studies have indicated that NRSF may act in a concentration-dependent manner, suggesting that low levels of NRSF may be insufficient for silencing activity (20, 32). To test this hypothesis and to get direct evidence of the role of NRSF in determining neuron-specific expression of the ChAT gene, we assayed different heterologous promoter constructs containing the ChAT NRSE after overexpression of human NRSF in FR3T3 and SN6 cells. In the first experiment, we used a construct containing two tandem ChAT NRSEs in front of the tk promoter. As expected, overexpression of NRSF in FR3T3 cells did not affect expression from this construct (Fig. 8*A*), consistent with the high levels of endogenous expression of NRSF already present in these cells. In contrast, overexpression of NRSF in SN6 cells repressed the activity of this promoter construct by 7-fold (Fig. 8*A*). NRSF overexpression had, however, no effect on the activity of the tk promoter alone (Fig. 8*A*), suggesting that direct binding of NRSF to the ChAT NRSE is required for repression of transcription. RPA of equal amounts of RNA from transfected cells demonstrated that the lack of effect of overexpressed NRSF in FR3T3 cells was not due to poor overexpression (Fig. 8*B*).

In the second experiment, we tested the effects of NRSF overexpression on the activity of heterologous promoter constructs containing two different segments of the 2336-bp regulatory region of the ChAT gene in SN6 cells. Constructs in which the 21-bp NRSE sequence had been deleted were also included for comparison. Overexpression of NRSF caused a statistically significant 3-fold reduction in the activity of the −1799 construct, containing 276 bp from the proximal part of the 2336-bp ChAT gene fragment including the NRSE (Fig. 9). More important, deletion of the NRSE in this sequence abolished the effects of NRSF overexpression (Fig. 9), confirming that NRSF activity requires an intact NRSE. Interestingly, NRSF overexpression had no effect on the activity of the −3859 construct, regardless of the presence or absence of the NRSE (Fig. 8), suggesting an interaction between upstream sequences in the ChAT gene promoter and the NRSE in cholinergic cells.

DISCUSSION

In this study, we report on the identification and functional analysis of positive and negative regulatory elements in a 2336-bp-long upstream region of the rat ChAT gene promoter capable of directing cell type-specific gene expression. We have previously demonstrated that this 2336-bp-long DNA sequence allows correct temporal and spatial expression of a reporter gene when linked to a heterologous tk promoter in transgenic mice (15). *In vitro*, this DNA fragment enhances the activity of a tk promoter in the cholinergic neuronal SN6 cell line, but suppresses expression from this promoter in the fibroblast cell line FR3T3 (15). Using deletion analysis, we have now localized the majority of the silencer activity of this fragment to its most 3′-region, where a 21-bp-long sequence highly related to a silencer element present in several other neuron-specific genes was found. This element, termed NRSE or RE1, has been found

to suppress non-neuronal expression of SCG10 (27), type II sodium channel (29), synapsin I (28), dopamine β-hydroxylase (30), and Na,K-ATPase α_3-subunit (33) genes. The ChAT NRSE differs from the NRSE consensus sequence (33) in two nucleotides located in positions known to vary among other NRSE sequences (Fig. 4). Interestingly, like all NRSE-containing promoters characterized so far, the ChAT gene promoter studied here lacks a TATA box (11).[2]

A nuclear protein that binds to the NRSE has recently been cloned (20, 32). This protein, termed NRSF or REST, contains eight zinc fingers and has been shown to bind the NRSEs of the SCG10, synapsin I, type II sodium channel, and BDNF genes (20, 32). We have shown here that NRSF is able to bind to the NRSE of the ChAT gene and to modulate gene promoter activity through this element. NRSF mRNA was found to be expressed in all three cell lines used for transfection studies, although the levels of NRSF mRNA in non-neuronal FR3T3 cells were substantially higher than in SN6 cells. Two other cholinergic lines of neuronal origin, MN1 and N2A, also expressed low levels of NRSF mRNA. *In vivo*, NRSF mRNA has been found in some subpopulations of embryonic neural crest-derived peripheral neurons (20, 32). Thus, although NRSF has been postulated to act as a repressor of gene expression in non-neuronal cells, it can also be found to some extent in neuronal cells. Furthermore, the presence of a NRSE-like element in the promoter of the BDNF gene, which is also expressed outside the nervous system, suggests that the mechanism whereby NRSF regulates gene expression may be more complex than previously anticipated. Thus, for example, it is conceivable that NRSF expression in embryonic neurons could reflect a role of NRSF in suppressing more mature neuronal phenotypes that should not be manifested until later stages of neuronal development. On the other hand, previous studies have indicated that NRSF may act in a concentration-dependent manner and that a threshold level of NRSF may be required for efficient repression of transcription (20, 32). Thus, the levels of NRSF found in some cells of neuronal origin may not be sufficient for transcriptional repression. In support of this hypothesis, we have found that overexpression of NRSF in SN6 cells did suppress expression from a NRSE-linked promoter, suggesting that endogenous levels of NRSF in these cells may be below the threshold required for silencer activity. The expression of NRSF in cells of neuronal origin could also reflect alternative roles for NRSF in these cells and suggests that neurons may express additional factors that either cancel or modify the effects of NRSF. Evidence for the existence of such a mechanism was obtained from the analysis of upstream sequences in the ChAT gene promoter.

An enhancing activity specific for cholinergic cells was also found in the 2336-bp upstream region of the ChAT gene. Deletion analysis of this region revealed several segments that cooperated to achieve this enhancing activity. In particular, the distal 500-bp segment of this region, in combination with downstream sequences situated around −2500 bp, but not alone, was able to stimulate transcription in cholinergic cells. These upstream sequences conferred additional specificity to the activity of a NRSE-linked promoter, suggesting that they cooperate with the ChAT NRSE in directing expression to cholinergic neurons. From experiments with transformed *Drosophila melanogaster* flies, the *Drosophila* ChAT gene expression seems to be controlled through a similar interaction between negative and positive elements residing in 7.4-kilobase pair upstream sequence (13, 14). In the dopamine β-hydroxylase gene, a cooperation between the NRSE and a cAMP-responsive element appears to be required for tissue-specific expression (30). The fact that the enhancing effect of the upstream region of the ChAT gene appeared to be confined to SN6 cells suggests that it contains elements that may function as enhancers in specific subpopulations of neurons. Further evidence for an interaction between upstream and downstream regulatory elements in the ChAT gene promoter was obtained after overexpression of NRSF in cholinergic SN6 cells. The fact that excess NRSF did not affect the activity of a construct that included the upstream enhancing sequences from the ChAT gene promoter indicates that additional elements in this region may be able to recruit factors that prevent NRSF-mediated silencing. These factors may interact directly with the NRSF·NRSE complex or may simply prevent binding of NRSF to its cognate element. On the other hand, the absence of such interactions in non-cholinergic cells would allow NRSF suppressing activities to be fully manifested. Thus, NRSF appears to be just one component of the mechanism regulating neuron-specific gene expression, which seems to also depend upon the expression status of additional regulatory factors. The mammalian transcriptional regulator NF-κB has been shown to function either as a repressor or as an activator depending on the actions of the high mobility group-like proteins DSP1 and HMGI(Y) (34). Could NRSF act as a dual repressor/activator depending on the cell context? Our 3′-deletion analysis suggests that the ChAT NRSE is not required for the enhancing effects of upstream regulatory sequences of the ChAT gene, although it does not rule out that such a mechanism may be operating *in vivo*.

Taken together, our data reveal a modular arrangement of regulatory elements in the ChAT gene promoter that together determine the cell-type specificity of ChAT gene expression. At least two kinds of elements were found to cooperate to direct specific gene expression to cholinergic neurons, namely a neuron-restrictive silencer element and cholinergic-specific enhancer sequences. Our results suggest that similar arrangements of neuron-restrictive silencers and specific enhancers may be found in the promoter regions of other neuron-specific genes; this may constitute a general mechanism for the control of gene expression within specific subsets of neurons.

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