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## Is RE1/NRSE a Common *cis*-Regulatory Sequence for ChAT and VAcHT Genes?\*

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**Choline acetyltransferase (ChAT), the biosynthetic enzyme of acetylcholine, and the vesicular acetylcholine transporter (VAcHT) are both required for cholinergic neurotransmission. These proteins are encoded by two embedded genes, the VAcHT gene lying within the first intron of the ChAT gene. In the nervous system, both ChAT and VAcHT are synthesized only in cholinergic neurons, and it is therefore likely that the cell type-specific expression of their genes is coordinately regulated. It has been suggested that a 2336-base pair genomic region upstream from the ChAT and VAcHT coding sequences drives ChAT gene expression in cholinergic structures. We investigated whether this region also regulates VAcHT gene transcription. Transfection assays showed that this region strongly represses the activity of the native VAcHT promoters in non-neuronal cells, but has no major effect in neuronal cells whether or not they express the endogenous ChAT and VAcHT genes. The silencer activity of this region is mediated solely by a repressor element 1 or neuron-restrictive silencer element (RE1/NRSE). Moreover, several proteins, including RE1-silencing transcription factor or neuron-restrictive silencer factor, are recruited by this regulatory sequence. These data suggest that this upstream region and RE1/NRSE co-regulate the expression of the ChAT and VAcHT genes.**

In cholinergic neurons, the neurotransmitter acetylcholine is synthesized by choline acetyltransferase (ChAT)<sup>1</sup> and is then translocated into synaptic vesicles by the vesicular acetylcholine transporter (VAcHT). The expression of ChAT and VAcHT proteins is tightly linked. Both ChAT and VAcHT are absolutely required for cholinergic neurotransmission (1), and, in the nervous system, these proteins are only synthesized in cholinergic cells. Moreover, ChAT and VAcHT are both ex-

pressed from the same gene locus, referred to as “cholinergic gene locus.” Indeed, in nematode, *Drosophila*, and mammals, the VAcHT gene resides within the first intron of the ChAT gene and in the same transcriptional orientation (Fig. 1; Refs. 2–6).

The mode of transcription of these two unconventionally organized genes has been elucidated in the rat. In this species, the 5' end of the ChAT gene is composed of three non-coding exons, designated R, N, and M, that are alternatively contained into five different mRNAs (Fig. 1; Ref. 7). These transcripts are generated from several promoters, two of them lying upstream from exons R and M (Fig. 1; Refs. 8–10). Multiple promoter regions have also been identified for the VAcHT gene and give rise to five VAcHT mRNAs with different 5'-untranslated sequences. One promoter, located upstream from the exon R, may be common to the ChAT gene, whereas two VAcHT-specific promoters lie downstream from this exon (Fig. 1; Refs. 3 and 11). These various transcripts each encode single ChAT or VAcHT proteins. This unusual organization for two functionally related mammalian genes suggests that the ChAT and VAcHT genes may be co-regulated. Indeed, coordinated up-regulation of ChAT and VAcHT transcripts by various extracellular factors such as leukemia inhibitory factor, ciliary neurotrophic factor, retinoic acid, cAMP, glucocorticoids, nerve growth factor, and even stress has been demonstrated (12–19). Moreover, several studies have reported that, in most regions of the central nervous system, ChAT and VAcHT mRNAs and proteins have a comparable anatomical distribution and are co-localized within the same neurons (20–23). Therefore, the ChAT and VAcHT genes may share transcriptional regulatory sequences that restrict their expression to cholinergic neurons.

Transient transfection experiments previously revealed that the 3-kilobase pair genomic sequence upstream from the translation initiation site of VAcHT (regions 2 and 3, Fig. 2) does not contain such regulatory DNA sequences (8, 11). In contrast, various data suggest that a 2336-bp region just upstream and designated region 1 (Fig. 2) regulates the cholinergic neuron-specific expression of the ChAT gene. (i) In non-neuronal cells, *in vitro*, this region is able to repress the activity of the ChAT/VAcHT promoter region 2, as well as that of a heterologous promoter (8, 24); (ii) in transgenic mice, region 1 drove the expression of a downstream heterologous promoter in various cholinergic structures (25); (iii) the pattern of transgene expression in the brain of these mice paralleled both qualitatively and quantitatively that of endogenous ChAT mRNAs; and (iv) in spinal cord, transgene expression was developmentally regulated, similarly to the endogenous ChAT gene.

Region 1 contains a 21-bp sequence homologous to the neuron-restrictive silencer element (NRSE, Ref. 26) of the SCG10 gene or repressor element 1 (RE1, Ref. 27) of the type II sodium

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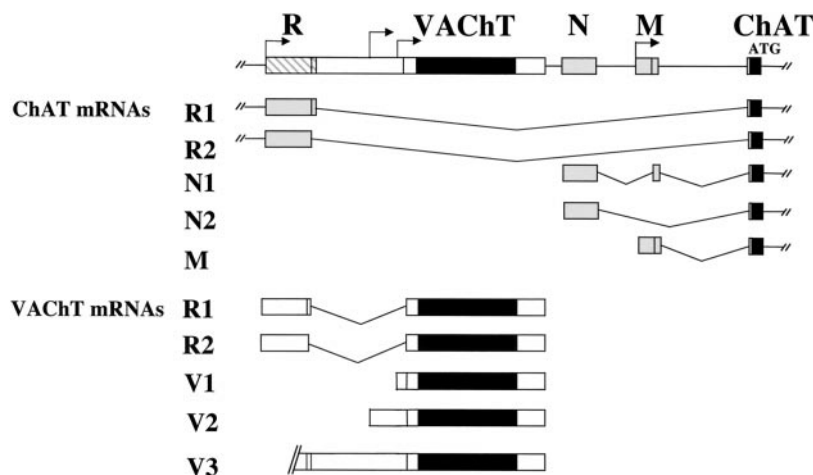
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<sup>1</sup> The abbreviations used are: ChAT, choline acetyltransferase; EMSA, electrophoretic mobility shift assay; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; RE1/NRSE, repressor element 1/neuron-restrictive silencer element; REST/NRSF, RE1-silencing transcription factor/neuron-restrictive silencer factor; tk, thymidine kinase; VAcHT, vesicular acetylcholine transporter; bp, base pair(s); RT, reverse transcription; PCR, polymerase chain reaction.

FIG. 1. Schematic representation of the rat cholinergic gene locus (*top line*) and of the various rat ChAT and VACHT mRNA species (7, 11). Black filled boxes indicate coding sequences; open and gray filled boxes represent non-coding sequences of the VACHT and ChAT genes, respectively. R, N, and M are the three non-coding exons of the ChAT gene. Exon R, which is shared by both ChAT and VACHT genes, is shown in gray and white. Vertical bars within the exons represent splice sites. Arrows indicate the transcriptional start sites identified for the ChAT and/or VACHT genes.



channel gene. A similar motif is present in the regulatory regions of several other genes whose expression is restricted to neurons (28). The transcription factor that binds RE1/NRSE is a zinc-finger repressor protein designated REST (for RE1-silencing transcription factor, Ref. 29) or NRSF (for neuron-restrictive silencer factor, Ref. 30). REST/NRSF mRNA and protein have been detected in a majority of non-neuronal cell types and in undifferentiated neuronal progenitors (29, 30), suggesting that REST/NRSF prevents full expression of the neuronal phenotype during early neurogenesis. In addition, REST/NRSF mRNA is differentially expressed in mature neurons of the adult brain (31). REST/NRSF may thus be also required for the differential expression of target genes in different neuronal subpopulations. The RE1/NRSE of region 1, *in vitro*, represses the expression of a heterologous downstream promoter in non-neuronal cells by binding REST/NRSF (24).

We investigated whether region 1 and more specifically RE1/NRSE are also involved in the cell-specific regulation of the VACHT gene expression in the rat. Transient transfections of non-neuronal and neuronal-like cells revealed that region 1 and RE1/NRSE are parts of a regulatory mechanism that strongly represses VACHT gene expression in non-neuronal cells, but not in neuronal cells. We also demonstrated the existence in non-neuronal cells of several nuclear proteins or protein complexes that interact specifically with this silencer element. Region 1 and RE1/NRSE may thus co-regulate both ChAT and VACHT gene expression. Moreover, the cholinergic specific expression of VACHT gene requires the contribution of additional control elements of the cholinergic gene locus.

#### EXPERIMENTAL PROCEDURES

**Reverse Transcription-PCR (RT-PCR)**—Total RNA was purified from indicated cell lines by the RNable method (Eurobio) and quantified spectrophotometrically. Single-stranded cDNAs were synthesized with avian myeloblastosis virus reverse transcriptase (Promega) using 1  $\mu$ g of RNA as the template and random hexamers (pd(N)<sup>6</sup>, Amersham Pharmacia Biotech) as primers. Reactions without RNA or without reverse transcriptase were used as controls. A Prolabo thermal cycler was used for PCR with one eighth of the reverse transcription reaction mix as template and Taq DNA polymerase (Promega), as described previously (12). The number of PCR cycles was 22 for G3PDH, 32 for VACHT, and 35 for ChAT and REST/NRSF cDNA amplifications. The sequences of the primers used are as follows: ChAT (+), GTCTTGGAT-GTTGTCATTAATTTTC; ChAT (-), TCTCTGGTAAAGCCTGTAGTAG-AGC; VACHT (+), CACCAAACTGTTCGGAAGCGGTG; VACHT(-), GCAGCGAAGAGCGTGGCATAGTC; REST/NRSF (+), GAGTCTGAA-GAACAGTTTCGTACAT; REST/NRSF (-), CTTGAAGTTGCTC-CTATCCGCTGT; G3PDH (+), ACCACAGTCCATGCCATCAC; G3PDH (-), TCCACCACCCTGTGCTGTA.

**Plasmid Constructions and Purification**—Various fragments of the promoter region of the rat VACHT gene (Fig. 2) were subcloned into the

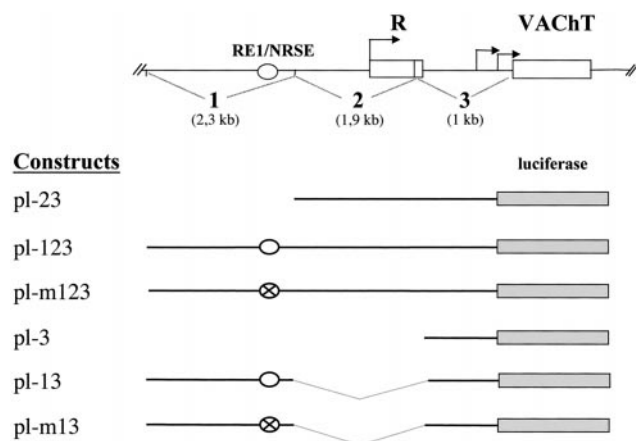
promoterless plasmid KSluc, upstream from the luciferase reporter gene and downstream from a transcription terminator. The KSluc was constructed by W. Faust and A. M. Catherin.<sup>2</sup> To simplify the plasmid nomenclature, we designated as 1, 2, and 3 the 2336-bp *EcoRI/HindIII* fragment upstream from the VACHT gene promoters, the 1895-bp *HindIII/HindIII* fragment containing the ChAT/VACHT promoter upstream from exon R, and the 1026-bp *HindIII/SphI* fragment containing the two VACHT promoters downstream from exon R, respectively (Fig. 2). DNA fragments 2, 3, 23, and 123 were prepared by appropriate digestion of a larger *EcoRI/EcoRI* genomic fragment of 7334 bp, obtained from a rat ChAT/VACHT genomic  $\lambda$ EMBL3 clone isolated previously (9) and subcloned into pBSKS<sup>+</sup> (Bluescript; Stratagene, La Jolla, CA). These fragments were inserted into the unique *HindIII* site of KSluc to generate the reporter plasmids designated pl-2, pl-3, pl-23, and pl-123, respectively. The plasmid pl-m123, which contains a two-base substitution mutation in the RE1/NRSE sequence, was generated from pl-123 by mutagenesis using the CLONTECH Transformer site-directed mutagenesis kit. The plasmids pl-13 and pl-m13 were obtained from pl-123 and pl-m123, respectively, by excision of fragment 2 by *HindIII* digestion, followed by self-ligation of the plasmids. Cloning junctions and the RE1/NRSE mutation were confirmed by DNA sequence analysis.

Two complementary oligonucleotides (cgcgTCCAGCACCACGGA-CAGTTCCa and cgcgTGGAACTGTCCGTTGCTGGA) containing the sequence of the region 1 RE1/NRSE flanked by *MluI* restriction sites, were annealed. The resulting double-stranded oligonucleotide was inserted into the *MluI* site of the pPGK-luc vector, described in Ref. 32, upstream from the phosphoglycerate kinase (PGK) promoter, to generate pPGK-luc-1, or oligomerized four times in the same orientation to create pPGK-luc-4. The number and orientation of the RE1/NRSE were checked by PCR and sequencing.

Plasmid DNA was purified either by two successive equilibrium centrifugations in CsCl gradients followed by a 48-h dialysis against water, or by using the Jetstar plasmid purification system (Genomed). DNA was quantified spectrophotometrically and by scanning pictures of ethidium bromide-stained DNA after migration on 1% agarose gel.

**Cell Cultures, Transient Transfections, and Reporter Gene Assays**—The cell lines FR3T3 (Fisher rat fibroblast), N2A (mouse neuroblastoma), and NG108-15 (mouse neuroblastoma N18TG-2  $\times$  rat glioma C6BU-1) were grown at 37  $^{\circ}$ C in a 7% (NG108-15) or 5% (N2A, FR3T3) CO<sub>2</sub> atmosphere in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) with 10% fetal calf serum (ATGC, Inc.). For NG108-15, the culture medium was supplemented with 1 mM sodium pyruvate, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, and 1  $\times$  HAT solution (100  $\mu$ M hypoxanthine, 0.4  $\mu$ M aminopterin, 16  $\mu$ M thymidine). Transfections were performed by the calcium phosphate precipitation method (N2A and NG108-15) or by electroporation with a single electrical pulse at 240 V using a Bio-Rad Gene Pulser (FR3T3). Cells (10<sup>6</sup>) were transfected with mixtures of 1 pmol of each reporter plasmid, 4  $\mu$ g of a SV40 promoter-chloramphenicol acetyltransferase vector (pCAT3-Control Vector, Promega) as an internal control to correct for differences in transfection efficiency, and a carrier DNA (pBluescript, Stratagene) to give a total amount of 12  $\mu$ g of DNA. For N2A and NG108-15 cells, medium was changed 16 h after transfection. Cells were harvested 40 h

<sup>2</sup> W. Faust and A. M. Catherin, unpublished results.

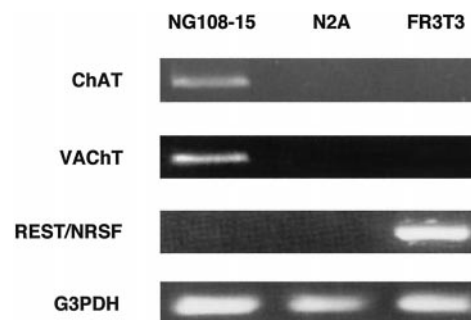


**FIG. 2. Schematic representation of the rat VAcHt gene (top line) and of the reporter gene constructs used for transfections that contain fragments of the VAcHt gene promoter region.** Boxes represent exon R and the VAcHt coding region. Arrows indicate the transcription initiation sites of the VAcHt gene. Regions 1, 2, and 3 were defined as follows: region 1, from  $-5605$  to  $-3269$  (0 corresponds to the position of the translation initiation site of VAcHt), between *EcoRI* and *HindIII* restriction sites; region 2, from  $-3268$  to  $-1373$  (restriction sites *HindIII* and *HindIII*); region 3, from  $-1372$  to  $-346$  (restriction sites *HindIII* and *SphI*). Region 2 contains the promoter region common to the ChAT and VAcHt genes, and region 3 the two specific promoters of the VAcHt gene. The open circle represents the RE1/NRSE homologous sequence. The crossed circle and *m* indicate that the RE1/NRSE is mutated. These VAcHt gene 5' regions were inserted into the plasmid KSluc upstream from the reporter gene encoding firefly luciferase.

after transfection. Luciferase activity was normalized with chloramphenicol acetyltransferase activity determined in the same extract (33). For each cell line, five to eight independent transfection experiments were performed. In each experiment, constructs were transfected in triplicate. For each construct, at least three independently prepared plasmid DNA samples were used. When the normalized activities driven by different constructs were compared, all the plasmids were prepared the same day.

**Statistical Analysis**—The data were analyzed by using the SAS statistical software (SAS Institute Inc., Cary, NC). Differences between the different groups (cell lines and/or constructs) were tested by analysis of variance (SAS/GLM procedure). The *p*-values corresponding to the two by two comparison were also computed (SAS/Ismeans and pdiff options; the test corresponds to the standard *t* test when no more than one explanatory factor is present). The homogeneity of the results according to the DNA preparation and the transfection experiment were tested by introducing these variables as explanatory factors into the analysis of variance models. Results obtained with small subsamples were checked by non-parametric tests.

**Nuclear Extracts and Electrophoretic Mobility Shift Assays (EMSA)**—Nuclear extracts were prepared from  $10^8$  cells as already described (34) with the following modifications: (i) in buffers A and C, EDTA and EGTA were omitted and  $ZnSO_4$  was added to a final concentration of  $1 \mu M$ , together with an additional mixture of protease inhibitors (0.1 unit/ml aprotinin,  $1 \mu M$  pepstatin,  $1 \mu M$  leupeptin, 1 mM iodoacetamide); (ii) nuclear extracts were dialyzed against buffer D (20 mM Hepes, pH 7.9, 250 mM KCl, 2.5 mM  $MgCl_2$ , 1 mM dithiothreitol,  $1 \mu M ZnSO_4$ , 25% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide). Protein concentrations were determined using the Bio-Rad assay system. The probe was prepared using a 49-mer double-stranded oligonucleotide, labeled with  $[\alpha\text{-}^{32}P]dCTP$  by Klenow filling-in, and purified following electrophoresis on a 8% polyacrylamide gel. The binding reaction was performed in a final volume of 20  $\mu l$  in buffer D containing 10% glycerol. Nuclear extracts (10  $\mu g$ ) were preincubated for 10 min on ice in buffer D complemented with 0.1% Nonidet P-40, 2  $\mu g$  of poly(dI-dC), and 50 ng of sonicated single-stranded herring sperm DNA. The  $^{32}P$ -end-labeled probe (10 fmol) was added either with or without a molar excess of a competitor oligonucleotide as indicated, and the binding reaction was continued for another 20 min at room temperature. For supershift experiments, either preimmune serum (1  $\mu g$ ) or 1 or 2  $\mu g$  of an anti-REST/NRSF polyclonal antibody (a generous gift from Dr. Gail Mandel, State University of New York, Stony Brook, NY) was preincubated with the binding reaction prior to addition of the probe.



**FIG. 3. RT-PCR analysis of ChAT, VAcHt, and REST/NRSF mRNA expression in various cell lines.** RNAs from NG108-15, N2A, or FR3T3 were reverse-transcribed, and PCR was performed as described under "Experimental Procedures" using primers specific for the coding regions of ChAT, VAcHt, RE1/NRSF, and, as control, G3PDH mRNA. The amplified products were separated on a 2% agarose gel containing ethidium bromide. The expected sizes of the PCR products are 707 (ChAT), 500 (VAcHt), 626 (REST/NRSF), and 452 bp (G3PDH). For each PCR, no band was detected in the absence of cDNA or with a cDNA synthesis mixture lacking reverse transcriptase.

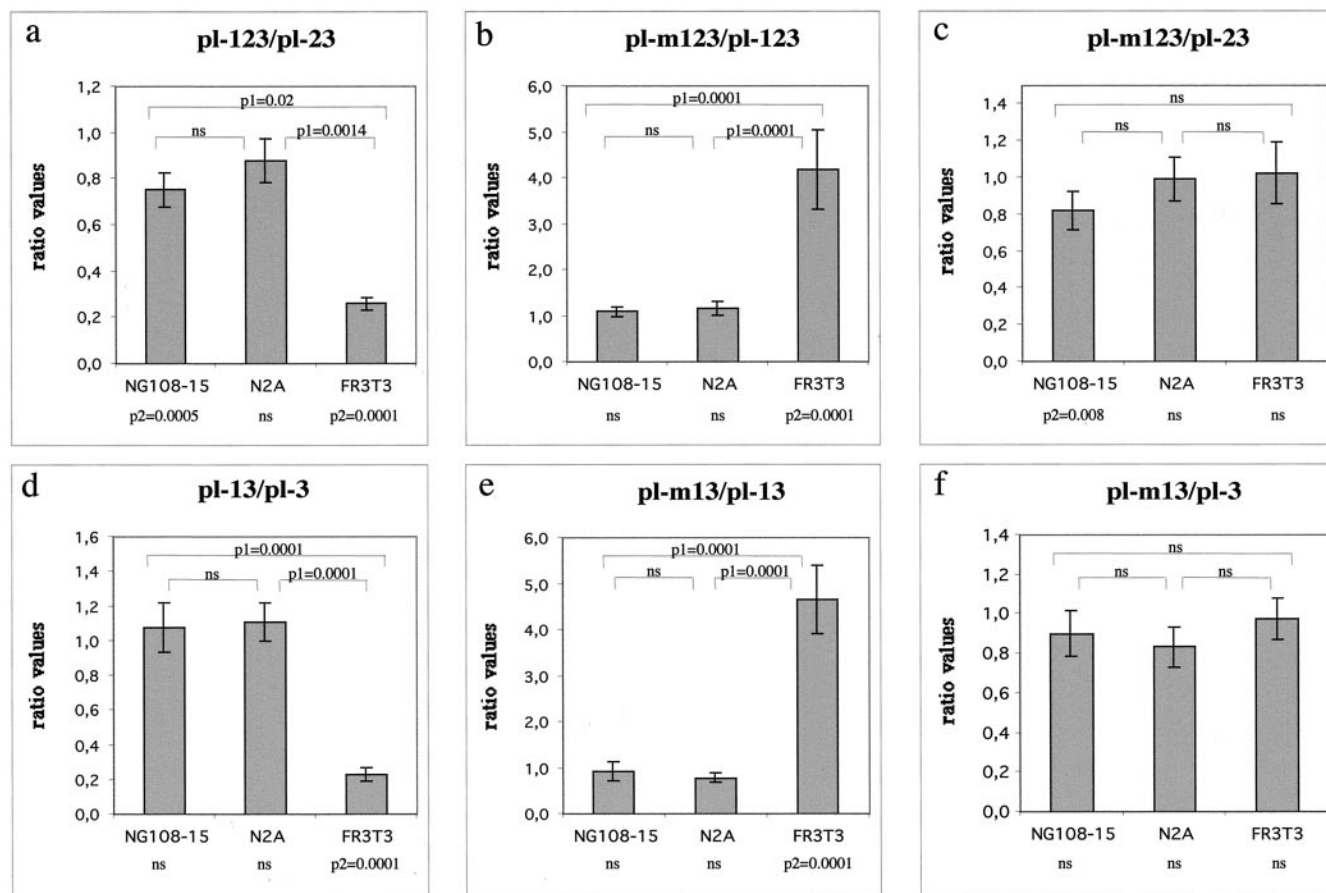
The DNA-protein complexes were resolved by electrophoresis on pre-run 6% native polyacrylamide gels in  $0.25 \times$  Tris borate/EDTA buffer. The following chemically synthesized double-stranded oligonucleotides with *SaI* overhangs were used as probes or competitors (unrelated nucleotides are shown in lowercase letters, and mutated nucleotides are underlined): wtRE1/NRSE, *tcgacTAGGAACTGTCCAGCACCACGGACAGTTCCGGGAGCCGCg*; mRE1/NRSE, *tcgacTAGGAACTGTCCAGCACCACCTACAGTTCCGGGAGCCGCg*; Y-TPH, *tcgacCCTTCTCAT-TGGCCGCTGCCAGCTg*.

## RESULTS

**Region 1 Is Involved in VAcHt Gene Cell Type-specific Expression**—Our previous studies have shown that the rat VAcHt gene can be transcribed from two distinct domains of the cholinergic gene locus: (i) one upstream from exon R (region 2, Fig. 2), which also promotes the transcription of the ChAT gene (3, 11); (ii) one downstream from exon R (region 3, Fig. 2), which contains two distinct promoters for the VAcHt gene. Both promoter regions 2 and 3 are constitutively functional in cholinergic cells, non-cholinergic neurons, and non-neuronal cells (11). Here, we used transient transfections to examine the potential involvement of a 5' upstream domain, region 1, in the regulation of the cell type-specific expression of the VAcHt gene.

Reporter plasmids were constructed, in which different combinations of the VAcHt promoter regions 2 and 3, with or without region 1, were fused to the firefly luciferase reporter gene (Fig. 2). These constructs were used to transfect three murine cell lines chosen for their different properties: NG108-15 (a cholinergic hybrid line), N2A (a non-cholinergic neuroblastoma line), and FR3T3 (a non-neuronal fibroblast line). The presence of ChAT and VAcHt mRNAs in NG108-15 was confirmed by RT-PCR experiments. These two transcripts were not detectable in N2A or FR3T3 (Fig. 3). These cell lines were also tested for the presence of REST/NRSF transcripts. In our PCR conditions, REST/NRSF mRNA was only detected in FR3T3 (Fig. 3), indicating that NG108-15 and N2A do not normally express high amounts of REST/NRSF and thus behave as neuronal cells.

Numerous independent transfection experiments were performed in NG108-15, N2A, and FR3T3 with different sets of DNA preparations. When cells from the same line were transfected simultaneously with different plasmid preparations of a given construction, the normalized luciferase activities obtained were variable and thus dependent on the DNA preparation, whatever the cell line. Therefore, to compare the transcriptional activities of different constructs, each transfection



**FIG. 4. Cell-specific repression of the VACHT gene promoter activity mediated by region 1 and RE1/NRSE.** NG108-15, N2A, and FR3T3 cells were transiently transfected as described under "Experimental Procedures" with the constructs shown on Fig. 2. Luciferase activity driven by each promoter region was normalized to chloramphenicol acetyltransferase activity from a co-transfected internal control plasmid that was measured in the same extract. To homogenize the results of several independent transfection experiments (see text), the normalized promoter activities were expressed as ratio values. Histograms represent the mean  $\pm$  confidence intervals (*error bars*) values for five to eight separate experiments performed with three or four series of independently prepared plasmid DNAs for each cell line. In each experiment, triplicate transfections were carried out. Ratio values were compared between cell lines by *t* test (*p*1). In each cell line, the normalized activities driven by the two constructs of each ratio were compared by analysis of variance adjusted for the DNA preparation series and the transfection experiment (*p*2). *ns*, not significant; *p* > 0.02.

experiment was carried out with plasmid DNAs that were all prepared at the same time and by the same protocol. However, in each cell line, the order of magnitude of normalized activities of the constructs varied from one experiment to another, whereas the ratio values between the normalized activities of two constructs were similar in the different experiments. We thus present the results of the normalized luciferase activities measured in several independent transient transfection experiments as ratio values obtained with different constructs. On the basis of these results, two different statistical analysis were performed (Fig. 4).

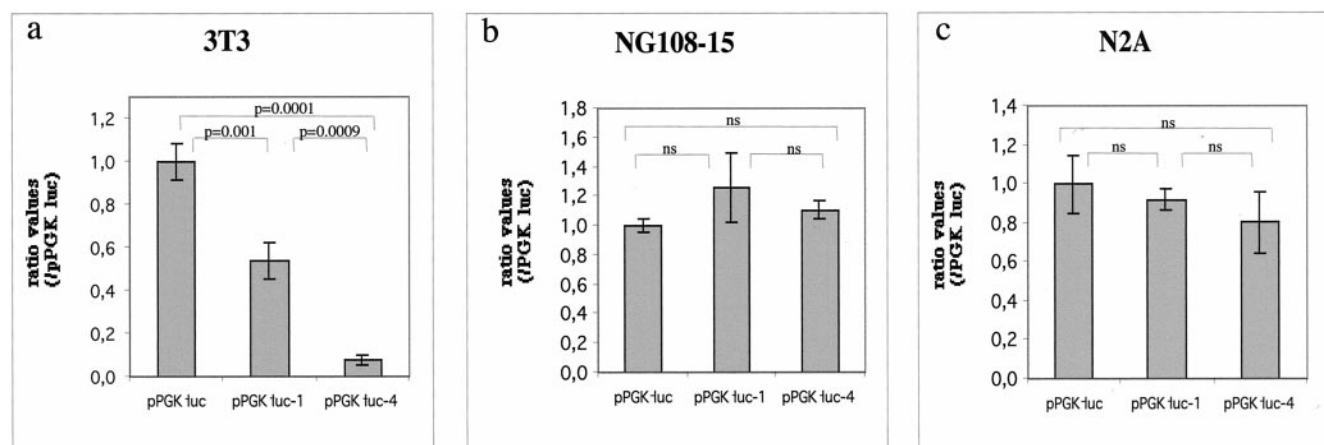
We first verified in NG108-15, N2A, and FR3T3 our previous observation in other cells (11) that the VACHT promoter regions 2 and 3 can be constitutively transcribed. This confirmed that both regions lack the regulatory motifs responsible for the cholinergic cell-specific expression of the VACHT gene.

We next examined the influence of region 1 on the transcriptional activities of the VACHT promoter regions 2 and 3. As shown on Fig. 4 (A and D) (ratios pl-123/pl-23 and pl-13/pl-3), the effect of region 1 in neuronal cells was significantly different from that in fibroblastic cells, whereas there was no major difference between its effects in cholinergic and non-cholinergic neuronal cells. In the NG108-15 cell line, transcription from the VACHT gene promoters of regions 2 and 3 together was slightly lower (25%) in the presence than absence of region 1 (Fig. 4A). This reduction was not observed when region 1 was

placed directly adjacent to region 3 (Fig. 4D). In N2A cell line, region 1 had no significant effect on the transcriptional activity of the whole VACHT promoter region 23 (Fig. 4A), or on that of region 3 alone (Fig. 4D). Region 1 is thus ineffective in these cells.

In contrast, region 1 substantially down-regulated the downstream VACHT promoters in fibroblastic cells FR3T3; the promoter activities of regions 23 and 3 were about 4-fold lower in the presence than the absence of region 1 (Fig. 4, A and D). Thus, this regulatory region exhibits a silencer function on the VACHT gene promoters, but only in non-neuronal cells. Note, however, that region 1 by itself was not sufficient to completely abolish these promoter activities in FR3T3 cells in our experimental conditions.

*The RE1/NRSE Motif Accounts for the Region 1-mediated Neuron-specific Expression of the VACHT Gene*—Region 1 contains a sequence homologous to RE1/NRSE (24), and this sequence may be responsible for repression of VACHT gene transcription in non-neuronal cells. The contribution of RE1/NRSE was explored by introducing point mutations into its sequence in the construct pl-123 and the chimeric construct pl-13. Two juxtaposing guanines near the center of RE1/NRSE were replaced by thymines. This mutation was previously shown to inactivate the RE1/NRSE motif of the SCG10 gene by abolishing the DNA binding of the repressor protein and thus its silencing activity (26).



**FIG. 5. Cell-specific repression of a heterologous PGK promoter mediated by RE1/NRSE.** Cells were transfected as described under “Experimental Procedures” by a luciferase expression plasmid in which no (pPGK-luc), one (pPGK-luc-1), or four tandem (pPGK-luc-4) copies of the ChAT/VAcHT gene RE1/NRSE motif were present upstream from the heterologous PGK promoter. Data were normalized as described in Fig. 4. Normalized luciferase activities are expressed relative to that driven by the plasmid containing no RE1/NRSE sequence (pPGK-luc). Histograms show mean  $\pm$  confidence intervals (error bars) of the results of one triplicate transfection representative of two or three independent experiments for each cell line. Means were compared two by two by *t* test. The ratios pPGK-luc-1/pPGK-luc and pPGK-luc-4/pPGK-luc were significantly different in non-neuronal cells and in neuronal cells (for pPGK-luc-1/pPGK-luc,  $p = 0.0001$  between FR3T3 and NG108-15;  $p = 0.005$  between FR3T3 and N2A; for pPGK-luc-4/pPGK-luc,  $p = 0.0001$  between FR3T3 and NG108-15 or N2A).

As expected, in neuronal cell lines, RE1/NRSE mutation did not significantly affect the strength of the VAcHT gene promoters. In both NG108-15 and N2A, the activities of the constructs pl-m123 and pl-m13 were similar to those of constructs pl-123 and pl-13, respectively (Fig. 4, B and E). In addition, the values of the ratios pl-123/pl-23 and pl-m123/pl-23 were significantly of the same magnitude (Fig. 4, A and C). Similarly, pl-13/pl-3 and pl-m13/pl-3 were not different in neuronal cells (Fig. 4, D and F). Thus, in N2A, region 1 in which RE1/NRSE has been inactivated is still without effect on VAcHT promoters. Moreover, in NG108-15, the faint region 1-mediated attenuation of the transcriptional activity of regions 2 and 3 is maintained (Fig. 4C), suggesting that this effect is independent of RE1/NRSE.

In contrast, mutation of the RE1/NRSE resulted in a tremendous derepression of transcription in fibroblastic cells. The promoter activities of constructs pl-123 and pl-13 were 4 times higher after mutation of this regulatory element (Fig. 4, B and E). In addition, the mutation restored fully the nonspecific promoter activities of regions 2 and 3: the ratios pl-m123/pl-23 and pl-m13/pl-3 were 1 (Fig. 4, C and F). Therefore, RE1/NRSE is a *cis*-acting sequence of the VAcHT gene involved in its repression in non-neuronal cells. Moreover, the silencing activity of region 1 in fibroblastic cells may not involve any cell-specific silencer elements other than the RE1/NRSE within this region.

The functionality of RE1/NRSE was then tested on a heterologous promoter in the cell lines used above. Plasmids were constructed, in which one or four tandem RE1/NRSE sequences in the same orientation are directly linked to the ubiquitous PGK promoter fused to the luciferase reporter gene. Transient transfection assays of these constructs showed that a single copy of this RE1/NRSE inhibited by 1.7-fold the activity of the PGK promoter in FR3T3 cells, whereas 4 tandem repeats repressed PGK promoter expression by 16.7-fold (Fig. 5). In contrast, there was no significant silencing effect of RE1/NRSE in the neuronal cell lines. These results are in agreement with those obtained with other cell lines and with the minimal thymidine kinase (tk) promoter and confirm that the biological activity of RE1/NRSE of region 1 is cell type-specific (24).

These data indicate that region 1 and RE1/NRSE contribute to the neuronal-specific expression of the VAcHT gene promoters. They also suggest that region 1 is not involved in the

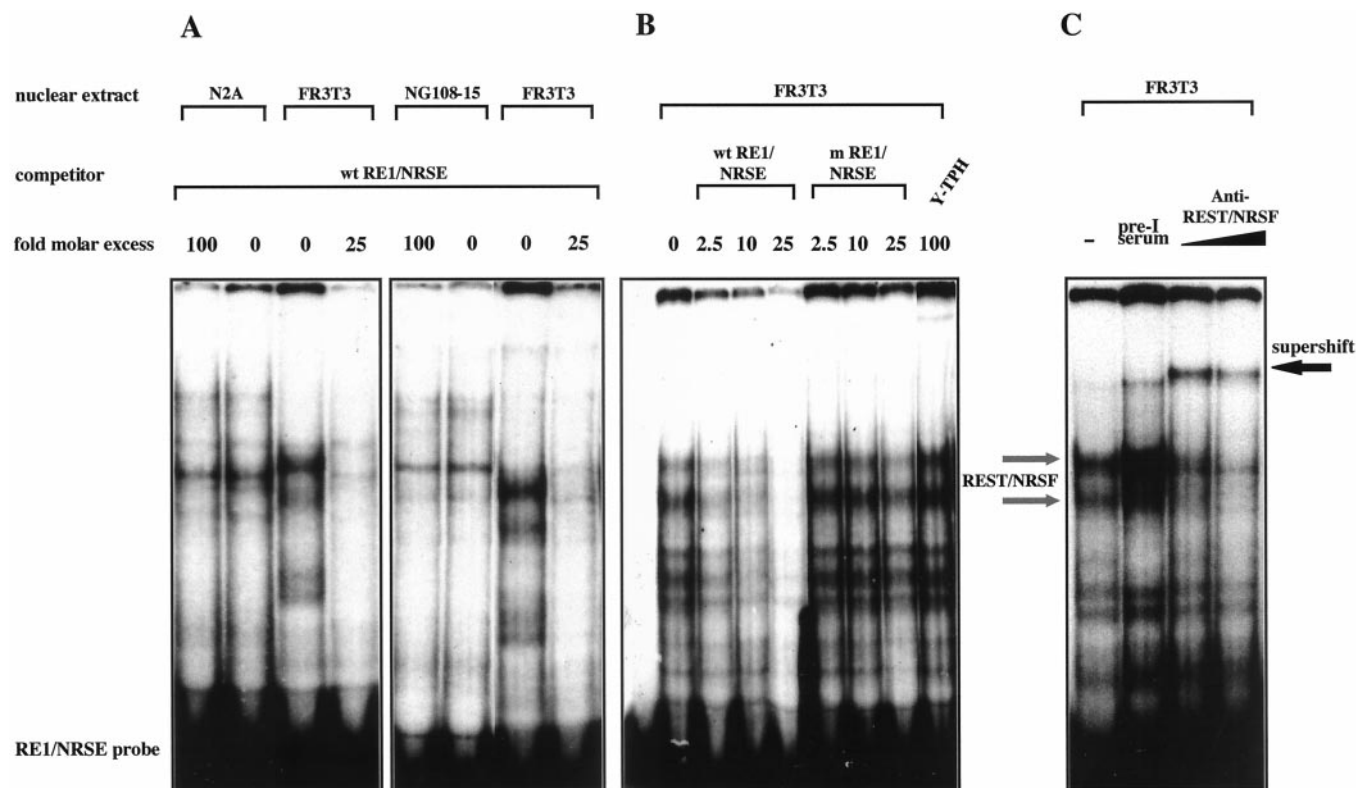
specific cholinergic expression of the VAcHT gene.

*Several Nuclear Protein Complexes Interact with RE1/NRSE*—EMSA and competition assays were performed to test whether the nuclei of the cells from the three lines used for transient transfections contain a RE1/NRSE binding activity. A radiolabeled 49-bp double-stranded oligonucleotide, wtRE1/NRSE, containing the sequence of the RE1/NRSE of region 1 with nine flanking nucleotides on either side, was used as a probe.

EMSA performed with nuclear extracts from NG108-15 or N2A revealed bands which appeared to be nonspecific since they were not competed by a 100-fold molar excess of the unlabeled wtRE1/NRSE oligonucleotide (Fig. 6A). The quality of the nuclear extracts used in these experiments was ascertained by testing protein binding to the probe Y-TPH containing the sequence of the inverted CAAT box of the human tryptophan hydroxylase gene (Ref. 33; results not shown). Thus, nuclear extracts of both neuronal cell lines do not contain a RE1/NRSE binding activity detectable by our assay. This is consistent with the lack of RE1/NRSE-mediated transcriptional repression of the VAcHT gene in these cell lines, and the absence of REST/NRSF mRNA.

In contrast, in the same experimental conditions, several specific DNA/protein complexes with different mobilities were clearly visualized with independent preparations of FR3T3 nuclear extracts (Fig. 6B). These complexes were also formed with the RE1/NRSE motif of the SCG10 gene (data not shown). The specificity of these complexes was established by a series of competition experiments. The signal for each of the complexes was abolished by the addition of unlabeled wtRE1/NRSE oligonucleotide. Competition was detectable with molar excess of wtRE1/NRSE as low as 2.5-fold. A 100-fold excess of an oligonucleotide with a sequence unrelated to RE1/NRSE (Y-TPH) did not reduce the signal for any of the DNA-protein complex. Moreover, an oligonucleotide containing the mutated RE1/NRSE motif (mRE1/NRSE) was much less effective than the wild type counterpart to compete for RE1/NRSE binding activity. This demonstrates that the complexes visualized with nuclear extracts of fibroblast cells are RE1/NRSE sequence-specific complexes.

We investigated whether the proteins bound to RE1/NRSE are immunologically related to REST/NRSF. EMSA experiments were performed with a polyclonal antibody raised



**FIG. 6. Nuclear proteins from FR3T3 cells, but not from NG108-15 and N2A cells, bind specifically to the RE1/NRSE sequence of region 1.** A and B, EMSA were performed using nuclear extracts (10  $\mu$ g) from NG108-15, N2A, or FR3T3 cells and the wild type RE1/NRSE-containing oligonucleotide (wtRE1/NRSE) as a probe. A, DNA binding experiments with nuclear extracts prepared from FR3T3, NG108-15, or N2A cells. Unlabeled wtRE1/NRSE oligonucleotide was included as a competitor in the molar excess indicated. B, DNA binding experiments with nuclear extracts of FR3T3 cells. Binding in the absence of competitor, or in the presence of the following unlabeled oligonucleotides added as competitors in the molar excess indicated, is shown: wtRE1/NRSE, the mutant oligonucleotide mRE1/NRSE or an unrelated oligonucleotide Y-TPH. C, EMSA were performed in the absence of antibody (-), and in the presence of either preimmune serum (pre-I) or an anti-REST/NRSF antibody. The RE1/NRSE-binding protein complexes indicated by gray arrows shift to the position shown by the black arrow.

against a 485-amino acid fragment of the human REST/NRSF protein that contains the zinc finger domain. Two major bands were supershifted by the antibody, whereas preimmune serum had no effect on probe mobility (Fig. 6C). Thus, at least two complexes involving REST/NRSF with an accessible antigenic site are recruited by RE1/NRSE.

#### DISCUSSION

Using transient transfections of non-neuronal and neuronal-like cell lines, we show that region 1, a 2336-bp genomic region located upstream from the rat VAcHT gene promoters, contributes to the neuron-specific expression of VAcHT. Region 1 was able to repress transcription initiated by the VAcHT promoters in the fibroblastic cells FR3T3, without affecting the activity of these promoters in neuronal cells that do not express the VAcHT gene. Mutation of a RE1/NRSE present in region 1 fully derepressed VAcHT promoter expression in non-neuronal cells, demonstrating that the silencing activity of region 1 is mediated by this element. This RE1/NRSE motif was also able to block transcription from a heterologous PGK promoter in a cell type-specific manner. These findings correlate well with the specific binding of nuclear proteins extracted from non-neuronal cells to the native RE1/NRSE.

Various experimental data already indicate that region 1 is a regulatory region for the ChAT gene (see Introduction). In particular, the pattern of expression of a transgene under the control of region 1 *in vivo* paralleled that of the ChAT gene in various regions of the adult rat central nervous system and in the developing spinal cord (25). In view of our findings and the organization of the ChAT and VAcHT genes as embedded genes with intermingled promoters, it seems very likely that region 1

is a common regulatory domain of both genes. Li *et al.* (35) localized a genomic domain of the human cholinergic gene locus that negatively regulates the activity of the more downstream ChAT specific promoter in non-cholinergic cells, and which contains the human homolog of the RE1/NRSE of region 1. Furthermore, this motif is functional on the tk promoter *in vitro* (36). It is thus likely that the RE1/NRSE motif of region 1 regulates the cell-type specific expression of the ChAT gene. Our demonstration that RE1/NRSE within region 1 is a functional cell-specific silencer element of the VAcHT gene is consistent with RE1/NRSE being a co-regulatory *cis*-acting sequence for both ChAT and VAcHT genes, providing thereby a mechanism to coordinate their expression.

However, region 1 and RE1/NRSE did not appear to be entirely sufficient to restrict the expression of the VAcHT gene in cholinergic neurons and even in neurons. In the presence of region 1, residual VAcHT promoter activity was detected in non-neuronal cells. This remaining transcriptional activity cannot be attributed to insufficient REST/NRSF in FR3T3 cells, since overexpression of REST/NRSF in these cells did not increase the level of transcriptional repression mediated by this factor (24). Therefore, region 1 and RE1/NRSE are not fully responsible for the neuron-specific expression of VAcHT. Moreover, in non-cholinergic neuronal cells that do not contain detectable REST/NRSF, region 1 did not significantly repress the transcriptional activity of the VAcHT gene promoters. This finding in the neuroblastoma cell line N2A has been confirmed in primary cultured noradrenergic sympathetic neurons, where

<sup>3</sup> F. Pajak, personal communication.

the construct pl-123 is as efficiently transcribed as pl-23.<sup>3</sup> Thus, the expression of the VAcHT gene specifically in cholinergic neurons is under the control of additional *cis*-elements elsewhere in the cholinergic gene locus. As the VAcHT promoters of regions 2 and 3 are constitutively active in all the three cell lines tested, it is likely that these regulatory elements provide binding sites for transcriptional repressors. In agreement with these suggestions, other regions of the cholinergic gene locus extending downstream from exon R have been shown to contribute by their silencing activity to the cell type-specific expression of the ChAT gene *in vitro* (35, 37, 38). The involvement of regulatory sequences between exon R and the first coding exon of the mouse ChAT gene in the cholinergic-specific targeting of gene expression has been confirmed in transgenic mice (39). The additional regulatory sites contained within this domain may act either independently or in concert with RE1/NRSE and REST/NRSF. The identification of such elements and of the regulatory proteins they bind would be of interest. It may reveal specific combinations of factors required for strict targeting of the expression of the VAcHT gene to cholinergic neurons.

The presence in region 1 of additional regulatory elements involved in the cholinergic specificity of gene transcription has been previously suggested on the basis of transient transfections with a heterologous promoter: a silencer domain, which decreased tk promoter activity specifically in non-neuronal cells, and a cholinergic enhancer region, which stimulated the activity of the tk promoter specifically in cholinergic cells (24). Our results with the native VAcHT promoters do not corroborate these data. In our experimental conditions, mutation of the RE1/NRSE motif abolished entirely the neuron-specific expression of the VAcHT promoters, suggesting the absence of any other silencer. Additionally, we never observed any increase in the activity of the VAcHT promoters in cholinergic cells when region 1 was linked upstream from region 3 or from region 23. On the contrary, a slight decrease of transcription driven by region 23 was observed. The reason this effect was not observed with the promoters of region 3 is not clear and may result from the juxtaposition of regions 1 and 3. These discrepancies between our and previous work may be explained by both the cell- and promoter-dependence of the regulatory activity of region 1. Indeed, the two cholinergic cell lines in which region 1 has been studied are SN6 (24) and NG108-15 (this study). Although, NG108-15 cells have been extensively used as a model to study the cholinergic neurotransmission and ChAT gene properties (10, 40, 41), they may not synthesize the same set of active forms of the regulatory factors as produced by SN6. Moreover, the studies of Lönnerberg *et al.* (24) were performed with a 156-bp heterologous minimal tk promoter. We analyzed the effect of region 1 on the native VAcHT promoter regions spanning 1–2.9 kilobase pairs of endogenous VAcHT gene sequences. Interactions with *trans*-acting factors bound to other regulatory elements of the VAcHT promoter regions 3 and 23 may mask the enhancing and silencing activities of region 1 when the tk promoter is used. The influence of both the cellular and promoter contexts on transcriptional regulation is also illustrated by the studies of the RE1/NRSE-elicited silencing effect as a whole. Depending on the reports, the degree of repression by a RE1/NRSE sequence varies from 2- to 50-fold in *in vitro* studies (26, 27, 42, 43). Our results also show that a single RE1/NRSE motif was 2.5-fold more efficient at repressing VAcHT promoters than the PGK promoter in FR3T3 cells. In transgenic mice models, it has been shown that mutation of the RE1/NRSE motif present in some genes results in ectopic activation of a reporter gene either in a very limited number of non-neuronal cells or in a larger spectrum of non-

neuronal cells (44–48). In addition, RE1/NRSE has been shown to behave either as a silencer or as an enhancer depending on the cellular context (46, 49).

As we have shown that RE1/NRSE efficiently represses the VAcHT promoters in non-neuronal cells, we tested for the nuclear binding activities that recognize this motif. EMSA analysis revealed several proteins or protein complexes in FR3T3 nuclear extracts that specifically interacted with RE1/NRSE. Two of these complexes showed a modified mobility in the presence of an antiserum raised against REST/NRSF. Conversely, nuclear extracts from NG108-15 and N2A lacked any specific binding activity. This is in good agreement with our PCR and transfection data. The presence of several different complexes is surprising in view of previous reports (24). Their detection here may be due to differences in the nuclear extract preparation protocols. The multiple shifted bands may either result from proteolysis or correspond to distinct protein complexes. They also may be due to the recruitment by RE1/NRSE of modified forms of REST/NRSF, for example by phosphorylation, splice variants of REST/NRSF (31), or factors closely related to or different from REST/NRSF. In addition, complexes that contain REST/NRSF may involve other interacting proteins. Indeed, REST/NRSF is a modular protein that contains two repressor domains, located at its N and C termini (50, 51), that interact with different nuclear factors to repress transcription (52). The mode of repression of the C-terminal domain is unknown, but the N-terminal domain has been shown to recruit the corepressor Sin3 and thereby histone deacetylase to silence neural specific genes, suggesting that REST/NRSF-mediated repression involves chromatin remodeling (53, 54). Several types of protein complexes are thus able to bind RE1/NRSE. This highlights the complexity of the mechanism by which RE1/NRSE regulates gene transcription.

In conclusion, we demonstrate that region 1 and RE1/NRSE regulate the VAcHT gene expression. In view of previous reports, this suggests that these regulatory region and element co-regulate both ChAT and the embedded VAcHT gene. Overall, investigations of the cell-specific transcription of these two genes reveal that the specific targeting of ChAT and VAcHT gene expression in cholinergic neurons seems to be tightly controlled by a highly complex mechanism that requires the interaction of several regulatory domains of the cholinergic locus.

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