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Regulatory region in choline acetyltransferase gene directs developmental and tissue-specific expression in transgenic mice

(development/gene promoter/gene regulation/silencer/spinal cord)

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ABSTRACT **Acetylcholine, one of the main neurotransmitters in the nervous system, is synthesized by the enzyme choline acetyltransferase (ChAT; acetyl-CoA:choline *O*-acetyltransferase, EC 2.3.1.6). The molecular mechanisms controlling the establishment, maintenance, and plasticity of the cholinergic phenotype *in vivo* are largely unknown. A previous report showed that a 3800-bp, but not a 1450-bp, 5′ flanking segment from the rat ChAT gene promoter directed cell type-specific expression of a reporter gene in cholinergic cells *in vitro*. Now we have characterized a distal regulatory region of the ChAT gene that confers cholinergic specificity on a heterologous downstream promoter in a cholinergic cell line and in transgenic mice. A 2342-bp segment from the 5′ flanking region of the ChAT gene behaved as an enhancer in cholinergic cells but as a repressor in noncholinergic cells in an orientation-independent manner. Combined with a heterologous basal promoter, this fragment targeted transgene expression to several cholinergic regions of the central nervous system of transgenic mice, including basal forebrain, cortex, pons, and spinal cord. In eight independent transgenic lines, the pattern of transgene expression paralleled qualitatively and quantitatively that displayed by endogenous ChAT mRNA in various regions of the rat central nervous system. In the lumbar enlargement of the spinal cord, 85–90% of the transgene expression was targeted to the ventral part of the cord, where cholinergic α-motor neurons are located. Transgene expression in the spinal cord was developmentally regulated and responded to nerve injury in a similar way as the endogenous ChAT gene, indicating that the 2342-bp regulatory sequence contains elements controlling the plasticity of the cholinergic phenotype in developing and injured neurons.**

The choice of a specific complement of neurotransmitters is a crucial step in the acquisition of a differentiated phenotype by developing neurons. The molecular mechanisms controlling the establishment and plasticity of neuronal phenotypes are only beginning to be understood, as more regulatory sequences and transcription factors affecting pathways of neuronal differentiation are being characterized. Acetylcholine, one of the main neurotransmitters in the nervous system, is synthesized by choline acetyltransferase (ChAT; EC 2.3.1.6), currently the most specific marker for cholinergic neurons. The most prominent cholinergic system consists of cells associated with cranial nerve nuclei and motor neurons of the spinal cord. In the forebrain, the basal forebrain complex contains the major cholinergic cell bodies. These have been implicated in cognitive functions (1) and in the pathogenesis of Alzheimer disease (2). The levels of ChAT enzyme activity and ChAT mRNA are developmentally regulated in the central nervous system (CNS) (3–5), and *in vivo* and *in vitro* studies have demonstrated that ChAT enzyme activity can be modulated by numerous extracellular effector molecules, including small hormones (6–9), cytokines (10), and growth factors (11–18).

In the rat, the gene encoding ChAT has been shown to span >64 kb and to contain 14 coding exons (19) and several alternatively spliced 5′ noncoding exons (20). Transcription of the rat and mouse ChAT genes appears to initiate at multiple promoters resulting in the production of five different 5′ mRNA sequences (20, 21). 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 (21–23). However, the molecular mechanisms regulating the establishment, maintenance, and plasticity of the cholinergic phenotype *in vivo* are largely unknown. We previously isolated a 3.8-kb segment from the rat ChAT gene promoter that directs expression of a reporter gene to cholinergic cells *in vitro* (22). Here, we show that a 2342-bp-long segment from this region confers cholinergic specificity on a noncholinergic promoter in an orientation-independent manner and directs correct developmental and tissue-specific expression to cholinergic cells in transgenic mice.

MATERIALS AND METHODS

DNA Cloning. A 2342-bp *Eco*RI–*Hin*dIII fragment from a genomic rat ChAT λEMBL3 clone (22) was isolated and subcloned into the *Sal* I site of pBLCAT$_2$ (24). This placed the 2342-bp fragment upstream of a herpes simplex virus thymidine kinase (*tk*) minimal promoter (spanning from −105 to +51) followed by the bacterial chloramphenicol acetyltransferase (CAT) reporter gene and polyadenylylation sequence from the simian virus 40 small-t-antigen gene.

Cell Culture, Transfection, and CAT Assay. FR3T3 fibroblasts and SN6 cells (25) were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) (GIBCO/BRL) 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 human neuroblastoma cell line N18TG2 and was kindly provided by David Hammond and Alfred Heller (University of Chicago). Cells grown to ≈70% confluency in 35-mm wells were transfected according to the calcium phosphate method with 1.5 μg of DNA plus 0.5 μg of a reference plasmid, pON260 (26), expressing β-galactosidase as internal control for transfection efficiency. After 48 hr, cells were harvested with phosphate-buffered saline containing 10 mM EDTA, transferred to 250 mM Tris (pH 7.5),

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Abbreviations: CAT, chloramphenicol acetyltransferase; ChAT, choline acetyltransferase; CNS, central nervous system; RT, reverse transcription; *tk*, herpes simplex virus thymidine kinase gene.

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and lysed by five cycles of freezing and thawing. CAT assays (27) used equal amounts of protein from each sample.

Transgenic Mice. A 4.2-kb fragment containing the 2342-bp *Eco*RI–*Hin*dIII fragment from the regulatory region of the rat ChAT gene followed by the *tk* minimal promoter, the CAT reporter gene, and polyadenylylation sequences was injected into pronuclei of fertilized mouse eggs to generate transgenic mice (28) (CBA × C57BL/6)F_1 mice were used as embryo donors, stud males, and pseudopregnant females. Mature C57BL/6 females were used for breeding. High molecular weight DNA from tail biopsies was prepared as described (28).

Sciatic Nerve Transection. Heterozygous transgenic mice, 3.5 weeks old, were deeply anesthetized with pentobarbital and both left and right sciatic nerves were transected at the mid-thigh level. Both the proximal and distal parts of ends of the axotomized nerves were reflected to minimize nerve regeneration. The operation did not prevent the mobility of the animals, which could readily access food and water supplies. Sham animals were similarly operated upon, but the sciatic nerve was not touched. Data were analyzed by Student's two-tailed *t* test for unpaired data.

Tissue Preparation, RNA Extraction, and Reverse Transcription (RT)–PCR Analysis. The age of embryos was determined by measuring crown–rump length. All tissues were immediately frozen on dry ice after dissection and stored at −70°C until analysis. The lumbar part of the cord was dissected and, while kept on dry ice, cut under the microscope into ventral and dorsal parts. For CAT assays, tissues were homogenized in 250 mM Tris (pH 7.5) with 20 strokes in a Dounce homogenizer. Samples were subsequently processed as described above for cultured cell lines. RNA was extracted (5) and RT–PCR was performed with 2 μg of total RNA according to manufacturer instructions (Perkin–Elmer).

RESULTS

Upstream Segment from the ChAT Gene Promoter Confers Cholinergic Specificity on a Noncholinergic Downstream Promoter *in Vitro*. Previous work identified a region of the ChAT gene promoter that directed gene expression to cholinergic cells *in vitro* (22). A 2342-bp 5′ flanking segment from this region, extending from an *Eco*RI site at −3865 to a *Hin*dIII site at −1523 (the transcriptional start site is defined herein as +1; P.L. and C.F.I., unpublished work), was subcloned in either orientation upstream of a *tk* minimal promoter driving expression of the bacterial CAT reporter gene. Both constructs were transfected into FR3T3 fibroblasts or into SN6 neuronal cholinergic cells, and transcriptional efficiency was measured by assaying CAT enzymatic activity. A construct with only the *tk* promoter and a promoterless construct were used as positive and negative controls, respectively. The *Eco*RI–*Hin*dIII fragment enhanced the activity of the *tk* promoter severalfold in SN6 cells as compared with the *tk* promoter alone (Fig. 1). This effect was observed regardless of the orientation of the *Eco*RI–*Hin*dIII fragment with respect of the downstream *tk* promoter. In contrast, transcription from the *tk* promoter in FR3T3 cells was greatly repressed by the *Eco*RI–*Hin*dIII fragment in either orientation (Fig. 1). This effect was due to the presence of specific silencer elements in this fragment (P.L. and C.F.I., unpublished work) and not to artifacts from vector sequences (29).

Regulatory Region in the ChAT Gene Promoter Directs Tissue-Specific Expression in Transgenic Mice. The heterologous construct containing the 2342-bp *Eco*RI–*Hin*dIII region upstream of the basal *tk* promoter and CAT reporter gene was microinjected into fertilized mouse eggs and transgenic lines were generated. Several other studies have demonstrated that the *tk* basal promoter is unable to direct cholinergic cell type-specific expression in transgenic mice when used alone or in combination with other regulatory sequences (30–32). Southern blot analysis identified 10 animals which had the transgene stably incorporated in their genome and that were able to transmit it to the offspring. Protein extracts from different brain regions and peripheral organs of F_1 animals were prepared and analyzed for CAT activity. Eight of the 10 lines were found to express the transgene. All 8 lines showed a similar regional distribution of CAT activity, as exemplified in Table 1 for the 3 lines displaying the highest levels of expression. However, expression levels did not correlate with transgene copy number (see legend to Table 1). High levels of CAT activity were found in the spinal cord of all expressing lines. In the brain of the 4-14 line, the levels and major sites of transgene expression correlated both qualitatively and quantitatively with areas previously shown to contain ChAT mRNA-expressing cells in the rat CNS (5), including pons, medulla, septum, olfactory bulb, and cortex (Fig. 2 *A* and *B*). Weak CAT activity in some of the brain regions represented true transgene expression as demonstrated by RT–PCR anal-

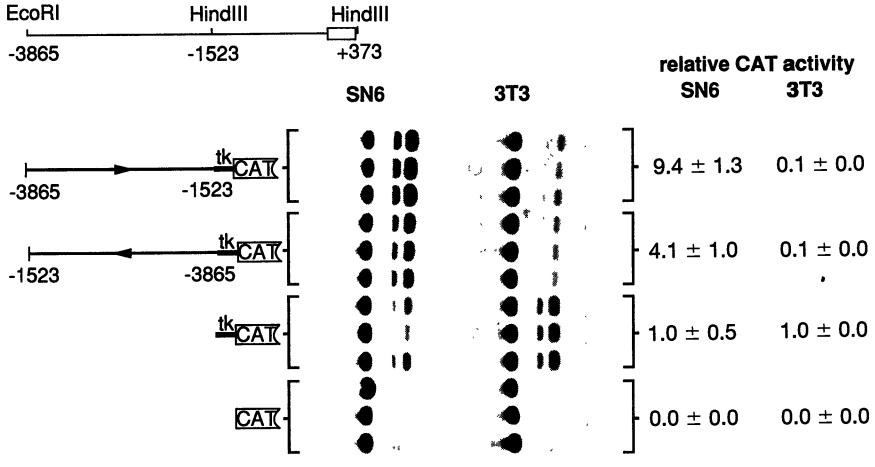

FIG. 1. Upstream 2342-bp *Eco*RI–*Hin*dIII ChAT gene segment confers cholinergic specificity on a heterologous *tk* promoter. Reporter gene expression was assayed as CAT activity in extracts of SN6 and FR3T3 cells transiently transfected in triplicate wells. CAT conversion values are given as mean ± SEM and are normalized to the value obtained with the *tk* promoter alone. A diagram of the upstream region of the ChAT gene is shown at the top; the 2342-bp *Eco*RI–*Hin*dIII fragment (shown as a thin solid bar in the lower diagrams) extends from position −3865 to −1523, transcription initiates at position +1, and the first exon of the ChAT gene is represented by an open box. Arrowheads in the 2342-bp enhancer segment indicate the relative orientations of this sequence with respect to the *tk* promoter (shown as a thick solid bar) and CAT reporter gene (open bar). The 156-bp sequence containing the *tk* promoter is not drawn to scale.

Table 1. Tissue distribution of CAT activity in three ChAT–CAT transgenic lines

Tissue	Relative CAT activity		
	3-13	4-14	4-17
Cortex	2.6	0.7	0.1
Cerebellum	4.7	1.2	0.0
Septum/striatum/thalamus/ hypothalamus	4.3	1.7	0.2
Pons/medulla/midbrain/ colliculi	9.5	5.1	2.4
Spinal cord	100	100	100
Thymus	2.6	1.5	0.6
Heart	0.0	0.0	0.1
Lung	0.1	0.0	0.1
Liver	0.8	0.1	0.1
Spleen	0.6	0.1	0.1
Pancreas	2.7	0.0	0.1
Duodenum	1.5	0.8	0.1
Kidney	0.1	0.1	0.0
Testis	0.3	0.1	0.0

Indicated tissues were dissected from 8-week-old mice from transgenic lines 3-13, 4-14, and 4-17. CAT assays used equal amounts of protein from homogenized extracts. Relative CAT activity in the spinal cord of each line was set to 100 (in this table and following figures, a relative CAT activity value of 100 in the adult spinal cord of lines 3-13, 4-14, and 4-17 corresponds to 1.5%, 2.2%, and 0.4% conversion of chloramphenicol per 10 μg of protein in 2 hr, respectively). Transgene copy number in lines 3-13, 4-14, and 4-17 was estimated at 1–2, 5–10, and 10–20, respectively, by Southern blot analysis (data not shown).

ysis (Fig. 2*C*). Transgene expression was also detected in areas known to receive cholinergic input, such as colliculi, thalamus, and hypothalamus (Fig. 2 *A–C*). No transgene expression was detected in the 4-14 line in hippocampus or striatum (Fig. 2 *A–C*). Overall, a similar pattern of transgene expression was observed in the brains of the 3 lines examined, with predominant expression in brainstem, basal forebrain, and cortex (Table 1). In 2 transgenic lines, CAT expression was detected in the cerebellum, a structure not previously shown to contain cholinergic neurons (Table 1). Various sites of weak peripheral expression were also observed in several transgenic lines, including thymus (5 of 8 lines), liver (5 of 8), kidney (3 of 8), duodenum (2 of 8), and pancreas and muscle (1 of 8) (Table 1, Fig. 2*C*, and data not shown). Interestingly, none of the 8 lines expressed the transgene in the testis, where germ cells have been shown to express ChAT mRNA at high levels (33).

To further establish the cholinergic origin of the transgene expression in the spinal cord, the lumbar enlargement of the spinal cord of transgenic animals was dissected and divided under the microscope into dorsal and ventral portions. For this experiment, the 3 lines showing highest expression of the transgene were chosen. Protein extracts were prepared and analyzed for CAT activity. In the 3 transgenic lines, 85–90% of the CAT activity was restricted to the ventral portion of the cord, where the cell bodies of cholinergic motor neurons are confined (Fig. 3). Together with the tissue distribution data, these results show that the 2342-bp regulatory region of the ChAT gene can direct cholinergic cell-specific expression from a noncholinergic promoter *in vivo*.

Transgene Developmental Regulation and Response to Nerve Injury Mimics Regulation of the Endogenous ChAT Gene. The developmental expression of the transgene was analyzed as CAT activity in spinal cords of animals from two different lines. The level of transgene expression increased slowly during embryonic development (Fig. 4). After birth, transgene expression increased more rapidly; the highest level was seen at 8 weeks of age, the latest time point examined (Fig. 4). Most importantly, transgene developmental regulation

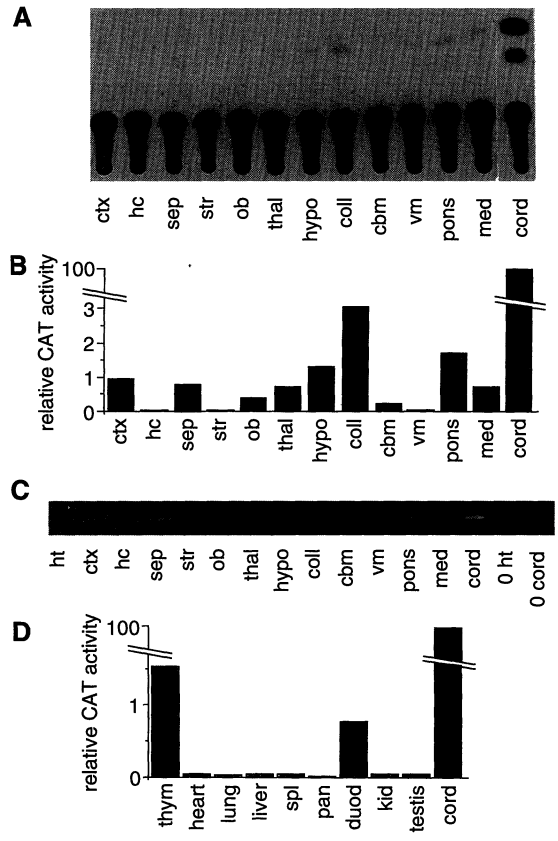

FIG. 2. Tissue-specific expression of CAT reporter gene in transgenic mice. (*A*) Autoradiogram of a CAT assay of tissue extracts from various brain regions from an 8-week-old transgenic mouse from line 4-14. ctx, Cortex; hc, hippocampus; sep, septum; str, striatum; ob olfactory bulb; thal, thalamus; hypo, hypothalamus; coll, colliculi; cbm cerebellum; vm, ventral midbrain; med, medulla; cord, spinal cord. (*B*) Quantitative distribution of transgene expression in the CNS of transgenic line 4-14. Relative CAT activity levels in spinal cord are defined here as 100. Abbreviations are as for *A*. (*C*) RT–PCR amplification of transgene mRNA in various brain regions from a transgenic mouse from line 4-14. Abbreviations are as in *A*, except ht, heart; 0 ht, nontransgenic heart; 0 cord, nontransgenic spinal cord. (*D*) Quantitative distribution of transgene expression in peripheral organs of transgenic line 4-14. Relative CAT activity levels in spinal cord (cord) are defined here as 100. thym, Thymus; spl, spleen; pan, pancreas; duod, duodenum; kid, kidney.

paralleled that of endogenous ChAT mRNA in rat spinal cord as analyzed by Northern blotting (Fig. 4).

Previous work showed a 2-fold increase in ChAT mRNA levels in spinal cord 7 days after a crush lesion of the sciatic nerve (5), probably reflecting part of a late regenerating response of motor neurons after injury. We therefore investigated the regulation of transgene expression in a paradigm of nerve damage. Bilateral sciatic nerve transection was performed in 3-week-old transgenic mice. CAT activity was assayed in protein extracts from the lumbar part of axotomized and sham-operated spinal cords 7 days after the operation. A significant 3- to 4-fold increase in CAT activity was observed in the axotomized spinal cord of transgenic animals compared with sham-operated controls (Fig. 5), suggesting that the promoter construct was also able to respond to nerve injury in a similar way as the endogenous gene.

DISCUSSION

We report that a 2342-bp regulatory region of the ChAT gene can confer cholinergic specificity on a downstream noncholinergic promoter in a cholinergic cell line and in transgenic

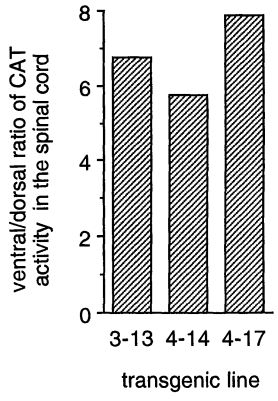

FIG. 3. Transgene expression is targeted to the ventral part of spinal cords from transgenic mice. The lumbar part of spinal cords of 8-week-old mice from the three indicated lines was dissected and divided into ventral and dorsal halves. The tissue was homogenized and CAT assays were performed. The ventral/dorsal ratio of CAT activity is shown for the indicated lines.

mice. This DNA segment enhanced the activity of a *tk* promoter in the cholinergic neuronal SN6 cell line but repressed expression from this promoter in the fibroblast cell line FR3T3. These effects were independent of the orientation of the 2342-bp fragment with respect to the *tk* promoter. These results suggest the presence of multiple regulatory elements that cooperate to control transcription initiation in cholinergic cells. In support of this hypothesis, we have recently identified a 21-bp silencer element related to an element previously shown to control neuron-specific expression of SCG10 and type II Na^+ channel genes in the proximal end of the 2342-bp fragment that is responsible for an important part of the silencer-like activity of this region in nonneuronal cell lines. In addition, other sequences localized to the same region appear to enhance the activity of a heterologous promoter in cholinergic cells. Sequence analysis of this region did not reveal any other sequence with similarity to previously described regulatory elements (P.L. and C.F.I., unpublished work). Thus, it appears that at least two kinds of regulatory elements in the ChAT gene are important for specific expression in cholinergic cells—namely, a neural-restrictive silencer element and a cholinergic-specific enhancer. The results of the present study further demonstrate that these elements are able to specifically direct gene expression to cholinergic regions of the CNS *in*

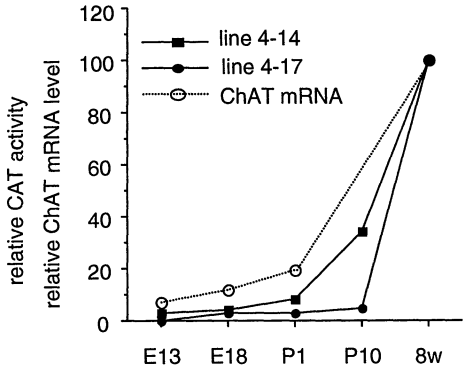

FIG. 4. Developmental regulation of transgene expression in the lumbar part of spinal cords from transgenic mice. Mice from transgenic lines 4-14 and 4-17 of the indicated ages were sacrificed and the lumbar part of the spinal cord was dissected, homogenized, and assayed for CAT activity. The developmental regulation of rat ChAT mRNA is shown for comparison (adapted from ref. 5). In the three curves, relative values at 8 weeks (8w) were set to 100, respectively. E13 and E18, embryonic days 13 and 18; P1 and P10, postnatal days 1 and 10.

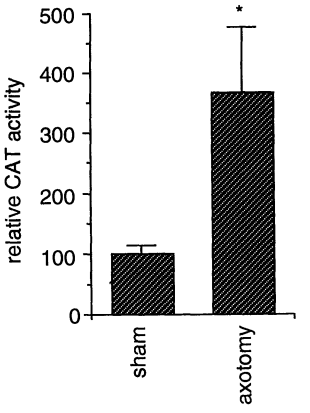

FIG. 5. Transgene expression in the spinal cord is increased after bilateral axotomy of the sciatic nerve. Sciatic nerves of 3-week-old mice from line 4-14 were bilaterally transected. Animals were sacrificed 7 days after the operation, and spinal cords were dissected, homogenized, and assayed for CAT activity. Bars show mean and SEM (*, $P < 0.025$; $n = 4$).

vivo. In all lines examined, the highest level of transgene expression was found in the spinal cord, followed by the pons and medulla (10- to 40-fold lower than spinal cord), nuclei of the basal forebrain, and cortex. The relative levels of transgene expression in CNS regions were in good agreement with the distribution of ChAT mRNA in the rat CNS (5). In contrast to previous reports in other rodents, a recent immunocytochemical study found no cholinergic cell bodies in mouse cerebral cortex (34). Basal forebrain cholinergic neurons, therefore, appear to provide the main cholinergic input to cortical cells in mice. CAT activity in cortex and in some other brain regions of ChAT–CAT transgenic mice, such as thalamus, hypothalamus, and colliculi, could therefore be the result of anterograde transport of the reporter gene product to terminals of cholinergic afferents, such as those from the basal forebrain and pedunculopontine tegmental nucleus. No transgene expression was detected in the striatum, which is known to contain sparse cholinergic interneurons. In the lumbar enlargement of the spinal cord, 85–90% of the transgene expression was targeted to the ventral part of the cord, where cholinergic α-motor neurons are located. Thus, the pattern of expression of the transgene agrees well both qualitatively and quantitatively with that reported for ChAT mRNA in various regions of the rat CNS.

The cholinergic phenotype of spinal cord motor neurons develops gradually during embryonic life. The level of ChAT mRNA increases slowly during embryonic development but more rapidly after birth, reaching maximal levels in the adult spinal cord (5). The transgene developmental regulation in the spinal cord mimicked that of the endogenous gene, indicating that control of tissue-specific expression and developmental regulation in the ChAT gene may be mediated by closely located, or even identical, elements and may therefore share common molecular mechanisms. Furthermore, the fact the transgene responded to nerve injury in a similar way as the endogenous gene suggests that these elements may also play an important role in the control of the cholinergic phenotype in injured neurons.

ChAT mRNA and protein have previously been detected in rat and human male germ cells (33), although the role of acetylcholine in sperm cell maturation and function remains to be established. None of the transgenic lines generated showed detectable levels of transgene expression in the testis. We have recently found that transcription initiates from the promoter region analyzed here in spinal cord and brain but not in testis (P.L. and C.F.I., unpublished work), suggesting the existence of alternative sites for the initiation of ChAT gene transcrip-

tion in testis. Previous reports indicated the presence of two additional promoters in the ChAT gene located downstream of the sequences studied here, from which transcription appears to also initiate in the spinal cord (20, 21). The individual contribution of the different promoters to ChAT gene expression in different tissues is unknown. Our results suggest that transcription from the ChAT gene in the testis may be differentially controlled from one of these alternative downstream promoters or from as yet uncharacterized regions of the gene. The regulatory region analyzed in this study may be useful to specifically target gene products to cholinergic neurons. This approach could prove useful for the generation of animal models for human diseases where degeneration of cholinergic neurons occurs, such as Alzheimer disease or amyotrophic lateral sclerosis.

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