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# Localization of Sequences Determining Cell Type Specificity and NGF Responsiveness in the Promoter Region of the Rat Choline Acetyltransferase Gene

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*Key words:* CAT assay, silencer element, SN6 cells, PC12 cells, cholinergic

## Abstract

A genomic clone containing 7 kb of 5′ flanking sequences from the rat choline acetyltransferase (ChAT) gene was isolated and shown to contain a TATA box-like sequence and several consensus binding sites for the transcription factor AP1. Two constructs containing 450 and 1450 base pairs (bp), respectively, of 5′ flanking sequences promoted expression of a fused chloramphenicol acetyltransfersase (CAT) gene when transfected into fibroblast FR3T3, Sertoli TM4, phaeochromocytoma PC12 and cholinergic neuronal SN6 cell lines. In contrast, a longer construct containing 3850 bp of 5′ flanking sequence allowed CAT activity only in the cholinergic cell line SN6. CAT activity with this construct was suppressed in the three other cell lines, indicating that the distal region of the ChAT promoter contains a cell type-specific silencer-like element that restricts ChAT gene expression to cholinergic cells. Treatment of PC12 cells with nerve growth factor (NGF) increased the promoter activity of the −450 and −1450 constructs approximately four-fold and allowed promoter activity from the −3850 construct, indicating that elements involved in NGF responsiveness of the ChAT promoter are contained in the first 450 bp of upstream sequence. These results support a model in which gene transcription controlled by cell-type specific regulatory elements contribute to the establishment, maintenance and plasticity of the cholinergic transmitter phenotype in the nervous system.

## Introduction

Choline acetyltransferase (ChAT; EC 2.3.1.6) is the enzyme responsible for the synthesis of the neurotransmitter acetylcholine. ChAT is mainly a nervous system-specific gene product, although some non-neuronal sources of ChAT have also been described, notably male germ cells (Bishop *et al.*, 1976; Ibáñez *et al.*, 1991a). Within the nervous system, ChAT expression is restricted to distinct subsets of neurons from both the peripheral and central (CNS) nervous systems, where it is considered a specific marker for cholinergic neurons. In the brain, the cholinergic system has been implicated in cognitive functions such as memory (Bartus *et al.*, 1982) and in the pathogenesis of Alzheimer's disease (Whitehouse *et al.*, 1982).

In the CNS, the levels of ChAT enzymatic activity and ChAT mRNA are developmentally regulated (Large *et al.*, 1986; Mobley *et al.*, 1989; Ibáñez *et al.*, 1991b). Both *in vivo* and *in vitro* studies have demonstrated that ChAT enzyme activity can be modulated by numerous extracellular effector molecules including retinoic acid (Matsuoka *et al.*, 1989), L-noradrenaline (Ishida and Deguchi, 1983), oestrogen (Luine *et al.*, 1986), thyroid hormones (Hefti *et al.*, 1986; Gould and Butcher, 1989), vitamin D (Sonnenberg *et al.*, 1986), interleukin-3 (Kamegai *et al.*, 1990) and basic fibroblast growth factor (Knusel *et al.*, 1990). In addition, several neurotrophic factors, including ciliary neurotrophic factor (Saadat *et al.*, 1989), nerve growth factor (NGF) (Honegger and Lenoir, 1982; Gnahn *et al.* 1983; Mobley *et al.*, 1986; Martínez *et al.*, 1987) and brain-derived neurotrophic factor (Knusel *et al.*, 1991; Alderson *et al.*, 1990) have been shown to increase ChAT enzyme activity in neuronal cultures. More recently, a 22-kD polypeptide named choline acetyltransferase developing factor (CDF) has been purified from rat skeletal muscle and shown to increase ChAT activity in cultures of embryonic rat spinal cord motoneurons (McManaman *et al.*, 1990). The stimulatory effect of NGF on ChAT activity is in line with several reports showing that NGF can prevent the loss of cholinergic neurons *in vivo* after specific brain lesions (Hefti, 1986; Williams *et al.*, 1986) and to decrease cholinergic neuron atrophy and spatial memory impairments in aged rats (Fischer *et al.*, 1987).

The molecular mechanisms by which neurotrophic factors and other transmembrane signalling agents control cholinergic function are largely unknown. Recently, NGF has been shown to increase the level of ChAT mRNA in rat septum as demonstrated by *in situ* hybridization (Higgins *et al.*, 1989) and the polymerase chain reaction (Cavicchioli *et al.*, 1991), suggesting that the stimulation of ChAT enzyme activity by NGF

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is due to an increase in the steady-state levels of ChAT mRNA. In this study, we report on the cloning and structure of upstream sequences of the rat ChAT gene and present evidence for elements within this sequence that confer cholinergic cell type specificity and NGF responsiveness on the ChAT promoter.

## Materials and methods

### *Cell culture*

The rat fibroblast cell line FR3T3, the rat Sertoli cell line TM4 (Mather *et al.*, 1982) and the cholinergic neuronal cell line SN6 (Hammond *et al.*, 1986) were grown in Dulbecco's modified essential medium supplemented with 10% fetal calf serum. The rat phaeochromocytoma cell line PC12 (Greene and Tischler, 1976) was grown in RPMI supplemented with 10% horse serum and 5% fetal calf serum. Differentiation of PC12 cells was obtained by addition of 200 ng/ml 7S NGF to the culture medium.

### *DNA cloning*

Approximately 1 000 000 clones from a rat (Sprague–Dawley) genomic library prepared by insertion of *Mbo*I-digested genomic DNA in the *Bam*HI site of phage λ EMBL-3 were screened with a double-stranded synthetic cDNA probe spanning 105 base pairs (bp) from the 5′ end of the published rat ChAT cDNA sequence (Brice *et al.*, 1989) labelled with α-[$^{32}$P]dCTP by fill-in with Klenow (Promega, Madison, WI) to a specific activity of $\sim 1 \times 10^9$ cpm/μg. Hybridization was carried out in 4× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 40% formamide, 1× Denhardt's solution and 10% dextran sulphate at 42°C. The filters were washed at 55°C in 0.1% sodium dodecyl sulphate (SDS) and exposed to Kodak XAR-5 films at −70°C. Four phage clones were isolated and a hybridizing 840-bp *Pst*I fragment from one of these clones was subcloned in the plasmid pBS-KS (Stratagene, La Jolla, CA). Sequence analysis (Sanger *et al.*, 1977) of the subcloned fragment confirmed the identity of the hybridizing region with that of the 5′ end of the rat ChAT sequence. For assays of promoter activity, different fragments containing 5′ flanking regions of the ChAT gene were subcloned in the *Xba*I–*Xho*I sites of pBLCAT$_2$ (Luckow and Schütz, 1987). A pBLCAT$_2$ plasmid containing the thymidine kinase (tk) promoter and a plasmid without promoter sequences were used in each experiment as positive and negative controls, respectively.

### *RNA preparation, RACE and Northern blot analysis*

Adult rat tissues were dissected from 150–200-g male Sprague–Dawley rats. The spinal cord and striatum were dissected as previously described (Ibáñez *et al.*, 1991b). Tissue samples were homogenized in 4 M guanidine isothiocyanate, 0.1 M β-mercaptoethanol, 0.025 M sodium citrate, pH 7.0, three times for 15 s with a Polytrone (Kinematica, Luzern, Switzerland). Each homogenate was layered over a 4-ml cushion of 5.7 M CsCl in 0.025 M sodium citrate, pH 5.5, and centrifuged at 15°C in a Beckman SW41 rotor at 35 000 r.p.m. for 16 h. RNA pellets were dissolved in 10 mM Tris–HCl, pH 7.5, 5 mM ethylenediamine tetraacetic acid (EDTA), 1% SDS, phenol–chloroform extracted and ethanol precipitated. Polyadenylated RNA (poly(A)$^+$ RNA) was purified by oligo (dT) cellulose chromatography and the recovery of RNA was quantified spectrophotometrically.

One μg of poly(A)$^+$ RNA from adult rat spinal cord, striatum and liver was used as a template for first-strand cDNA synthesis together with a synthetic oligonucleotide complementary to positions 219–238 of the rat ChAT cDNA sequence (Brice *et al.*, 1989) as specific primer (Frohman *et al.*, 1988). Excess primer was removed by ultrafiltration and the extended products were tailed with terminal deoxyribonucleotidyl transferase (IBI, New Haven) and dATP. The reactions were diluted to 550 μl with TE (10 mM Tris, pH 7.5, 1 mM EDTA), and 5 μl of each sample was used for polymerase chain reaction (PCR) amplification using a synthetic oligonucleotide complementary to positions 202–219 of the rat ChAT cDNA sequence and the RACE (rapid amplification of cDNA ends) primers as described by Frohman *et al.* (1988). One-tenth of each of these reactions was electrophoresed in a 1% agarose gel and transferred to a nitrocellulose filter. The filter was then hybridized with the synthetic cDNA probe used for the library screening, washed at 55°C in 0.1× SSC, 0.1% SDS, and exposed to a Kodak XAR-5 film at −70°C.

Spinal cord poly(A)$^+$ RNA (20 μg per lane) was electrophoresed in a 1% agarose gel containing 0.7% formaldehyde and transferred to nitrocellulose filters. The filters were then hybridized to consecutive restriction fragments from the isolated phage clone, as described in Results. The fragments were labelled with α-[$^{32}$P]dCTP by nick translation to a specific activity of approximately $5 \times 10^8$ c.p.m./μg. Hybridization was performed in 4× SSC, 40% formamide, 1× Denhardt's solution and 10% dextran sulphate at 42°C. Filters were washed at high stringency (0.1× SSC, 0.1% SDS, 60°C) and exposed to Kodak XAR-5 films at −70°C.

### *Transfection of cells and CAT assays*

Cells grown to ~70% confluency were transfected with 5 μg of plasmid DNA per 35-mm well using the calcium phosphate technique. Two days after transfection, cells were harvested in 0.25 M Tris, pH 7.5, and lysed by cycles of freezing and thawing. Endogenous acetyltransferases were inactivated by 10-min incubation at 65°C. Protein concentration in the different samples was equilibrated by measurement of absorbance at 280 nm. 40–60 μl of 200-μl extracts containing equivalent amounts of protein were assayed for chloramphenicol acetyltransferase (CAT) activity using standard procedures (Gorman *et al.*, 1982). The percentage of conversion of chloramphenicol into acetyl chloramphenicol obtained with each sample (which was never higher than 30%) was calculated after scintillation counting of the three chloramphenicol forms separated by thin-layer chromatography. These data were then used to calculate CAT activity in arbitrary units relative to the activity obtained with the Herpes simplex virus tk promoter, which was set to 10 units in each cell line. Background CAT activity, as measured using the construct without promoter, was below detectable levels in all cell lines tested.

## Results

### *Cloning and structure of 5′ flanking sequences of the ChAT gene*

Genomic sequences from the ChAT gene were isolated by screening a λ EMBL3 rat genomic library with a $^{32}$P-labelled double-stranded synthetic cDNA probe spanning 105 bp from the 5′ end of the published rat ChAT cDNA sequence (Brice *et al.*, 1989). The insert, consisting of one hybridization-positive phage clone, was mapped by restriction enzymes and the probe was shown to hybridize to the middle part of this 15-kilobase (kb) fragment (Fig. 1). Analysis of 2 kb of DNA sequence upstream from the hybridizing region revealed a TATA box-like sequence (TAAATAA) (Breathnach and Chambon, 1981) (Fig. 2)

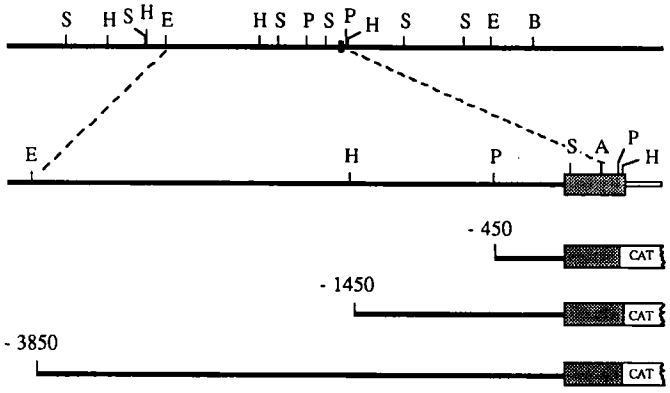


FIG. 1. Restriction map of the isolated 15-kb rat ChAT genomic clone. The small solid box indicates the location of the synthetic cDNA probe. Shown below is a schematic representation of the region analysed in this study. With the exception of the *Apa*I and *Pst*I sites, all other sites were completely mapped. Solid bar, 5′ flanking sequences; stippled box, first exon; open bar, intron sequences. The three constructs used for functional promoter assays are also shown. Abbreviations: A, *Apa*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sac*I.

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1   CTGCAGGGTATGTGATGGGCAGCTCTCTACTCAGATGAGTCTCTTCCTTTCAGGAGTACC
61  TGTGTACAGGTTGGGAGGTTGGGCACTAGACCACTTGATTGCTGCCTCTCTCCCTGACTC
121 TGTTCTCCATCACCTCCTCTTGCAACTGGGCTTAAGAACAGAAGCCAGCCAATTTTCCAG
181 CTCCTTCTGTATCTTCCACCATCCCAGACAGAGTCCAGGCTCACAATGCCTACCCAACTG
241 AGGAAGAAATCAGAGAGTCAGGATGCTCCCGGTGTCTGACTGCCCTTCACAAGACCCTCA
301 TGAACACAAGGCAGCAAGCACATGCTATAACAACAACGGCAAATGCTAATGATTCACACC
361 ACGCGTGTGCCACACCCTAGTTGTACGTACTCCTATTCCATTTTACAGATAAATAAAGGG
421 GCGGTGGGGGCAGAGGGAGGAAACAACGGCTCCCTTGGCACTGTTATCTAGTAAGTGGCA
                    Sac I
481 GCACTGGGAGCCATCCTATCTGTCTGCATCTGGAGCTCAAATCGTGATGCCTCCTTCGGT
541 GGAGGAAGGCTAGCTTGGGATATGGAGGCTACTGTGACTCCGGAAGACAGAGAAAAGTCC
601 AATCTCAACAACGTCACCACTATCCCCAATCTCAGCTGACTGGCATCCTCTCTCCTGCCA
661 GTCTGTGGACGGGAACGCGGGCCTCACGTGCCATCTAGGGTCAAAACTCGTCTGAGGACA
721 CACACTGGGCCCAACGCAGAGGCTGATCTGTTCAGCCTGTCGGCTGCAAGCCAGGACTCT
781 CAGCTTGTGCAGCACCCCCGGAAGGAAGGTGAGCCTTCCTAAGCCTCTACTGACAGCAAA
841 GCTGCAGAGGCCCTGCCGCGTGAGACCCAGAAGCTTCCAAGCCACTTGTGAGCCCACTCA
901 GGGTTTGGAGGCGGACGGGGTGGGGGTGGGGTGGGGAAA
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FIG. 2. Nucleotide sequence of the proximal regulatory region of the rat ChAT gene. Position 1 corresponds to the first nucleotide of the −450 construct (*Pst*I site). The sequence of the synthetic cDNA probe used for the library screening is underlined with a broken line. The exon−intron border is marked with an arrow. A TATA box-like sequence is boxed. AP1-like sites are underlined with a solid bar. The *Sac*I site marks the 5′ end of the most upstream fragment that hybridized with ChAT mRNA from rat spinal cord poly(A$^+$) RNA.

as well as eight AP1-like sites (Angel *et al.*, 1987) (note that only the four most 3′ AP1-like sites are contained in the sequence shown in Fig. 2). An exon−intron border was found 50 nucleotides downstream from the cDNA probe sequence (Fig. 2). Primer extension of

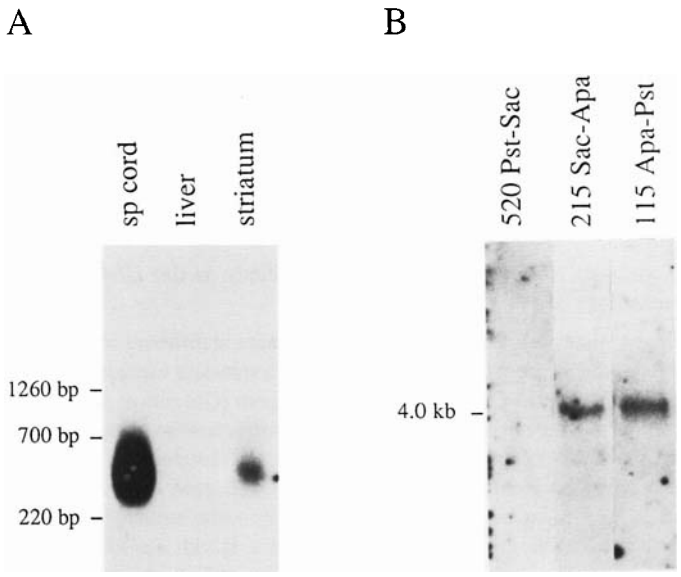


FIG. 3. Analysis of the 5′ end of the rat ChAT mRNA. (A) Autoradiogram of a Southern blot of the products of RACE amplification of RNA from the indicated rat tissues after hybridization to the synthetic cDNA probe used for library screening (see text for details). Shown in the Figure is an overnight exposure of the blot showing hybridizing signals in spinal cord and striatum. A shorter exposure (2 h) resulted in a signal in spinal cord which was similar to the one shown in the striatum. (B) Autoradiograms of Northern blots of rat spinal cord poly (A$^+$) RNA (20 μg per lane) after hybridization to the indicated restriction fragments from the isolated phage clone.

poly(A$^+$) RNA from rat spinal cord, striatum and liver (as control) followed by PCR amplification of the extended products was used to analyse the length of the 5′ end of the ChAT transcript (Frohman *et al.*, 1988). The specific primer used for PCR amplification was a synthetic oligonucleotide complementary to positions 202−219 of the rat ChAT cDNA sequence (Brice *et al.*, 1989) located 36 nucleotides downstream from the beginning of the second exon. The PCR amplified products were analysed by Southern blotting in combination with the synthetic cDNA probe used for the library screening. A specific band corresponding to a fragment of ~450 bp was detected in the spinal cord sample after a short exposure (Fig. 3A). Overnight exposure of the blot revealed a band of similar size in the striatum sample but not in the liver. This result indicated that the ChAT transcript extends at least 250 nucleotides upstream from the 5′ end of the published cDNA sequence. The region of the isolated ChAT genomic clone that is transcribed into mRNA was analysed by hybridization of rat spinal cord poly(A$^+$) RNA to consecutive restriction fragments from the isolated phage clone (Fig. 3B). A 115 bp *Apa*I−*Pst*I fragment (Fig. 1) containing the synthetic cDNA probe sequence detected a 4.0-kb ChAT transcript, in agreement with previous results using the entire rat ChAT cDNA as a probe (Ibáñez *et al.*, 1991b). The upstream, adjacent 215-kb *Sac*I−*Apa*I fragment (Fig. 1) also detected a specific ChAT mRNA of the same size. However, neither the next upstream fragment (520 bp *Pst*I−*Sac*I, Fig. 1) or consecutive upstream fragments in the next 7 kb of the clone detected ChAT mRNA, even after prolonged exposure times. These results are in agreement with the size of the extended products detected in spinal cord and striatum (Fig. 3A). Although this analysis cannot exclude the existence of additional short exonic segments further upstream and outside the phage clone, no sequences

other than those predicted by the genomic clone were found in the RACE products analysed. Taken together, these results indicate that the hybridizing fragments of the genomic clone correspond to the first exon of the rat ChAT gene, and that the transcriptional start site in rat spinal cord is in the vicinity of the *Sac*I site in the 215 bp *Sac*I–*Apa*I fragment (Figs 1 and 2). In agreement with this, the TATA box-like sequence is located 100 nucleotides upstream from this *Sac*I site (Fig. 2).

### *Distal sequences give cell type specificity to the ChAT promoter*

The promoter activity of the isolated sequences in different cholinergic and non-cholinergic cell lines was tested in a transient expression assay using the bacterial CAT gene as a reporter gene (Gorman *et al.*, 1982). For this purpose, three different plasmid constructs were made (Fig. 1). An 841-bp *Pst*I fragment containing ~450 nucleotides of 5′ flanking sequence was inserted upstream of the CAT gene in the plasmid pBLCAT$_2$ (Luckow and Schütz, 1987). The same strategy was used to insert a 1896-bp *Hin*dIII fragment and a 4.3 kb *Eco*RI–*Hin*dIII fragment containing 1450 and 3850 nucleotides of 5′ flanking sequence, respectively (Fig. 1). In addition to the 5′ flanking sequences, all three constructs included ~400 bp from the first exon of the ChAT gene (almost the entire exon) in front of the report gene. A pBLCAT$_2$ plasmid containing the tk promoter and a plasmid without promoter sequences were used as positive and negative controls, respectively. The various constructs were transfected into four different cell lines: FR3T3, a rat fibroblast cell line; TM4, a rat Sertoli cell line (Mather *et al.* 1982); PC12, a rat phaeochromocytoma cell line (Greene and Tischler, 1976) which shows low but significant ChAT activity and differentiates into a neuron-like cell after NGF treatment (Greene and Rein, 1977); and SN6, a cholinergic neuronal cell line derived from a fusion of mouse primary septal neurons with the human neuroblastoma cell line N18TG2 (Hammond, *et al.*, 1986). Hybridization of poly(A$^+$) RNA from these cells with the rat ChAT cDNA probe (Ibáñez *et al.*, 1991b) revealed high levels of a 4.0-kb ChAT mRNA in SN6 cells and lower levels in PC12 cells (data not shown). No ChAT mRNA could be detected in the FR3T3 or TM4 cells.

Both the −450 and −1450 constructs promoted transcription of the reporter gene in all cells tested (Fig. 4B), strongly indicating that the isolated sequences contain the promoter region of the ChAT gene. In

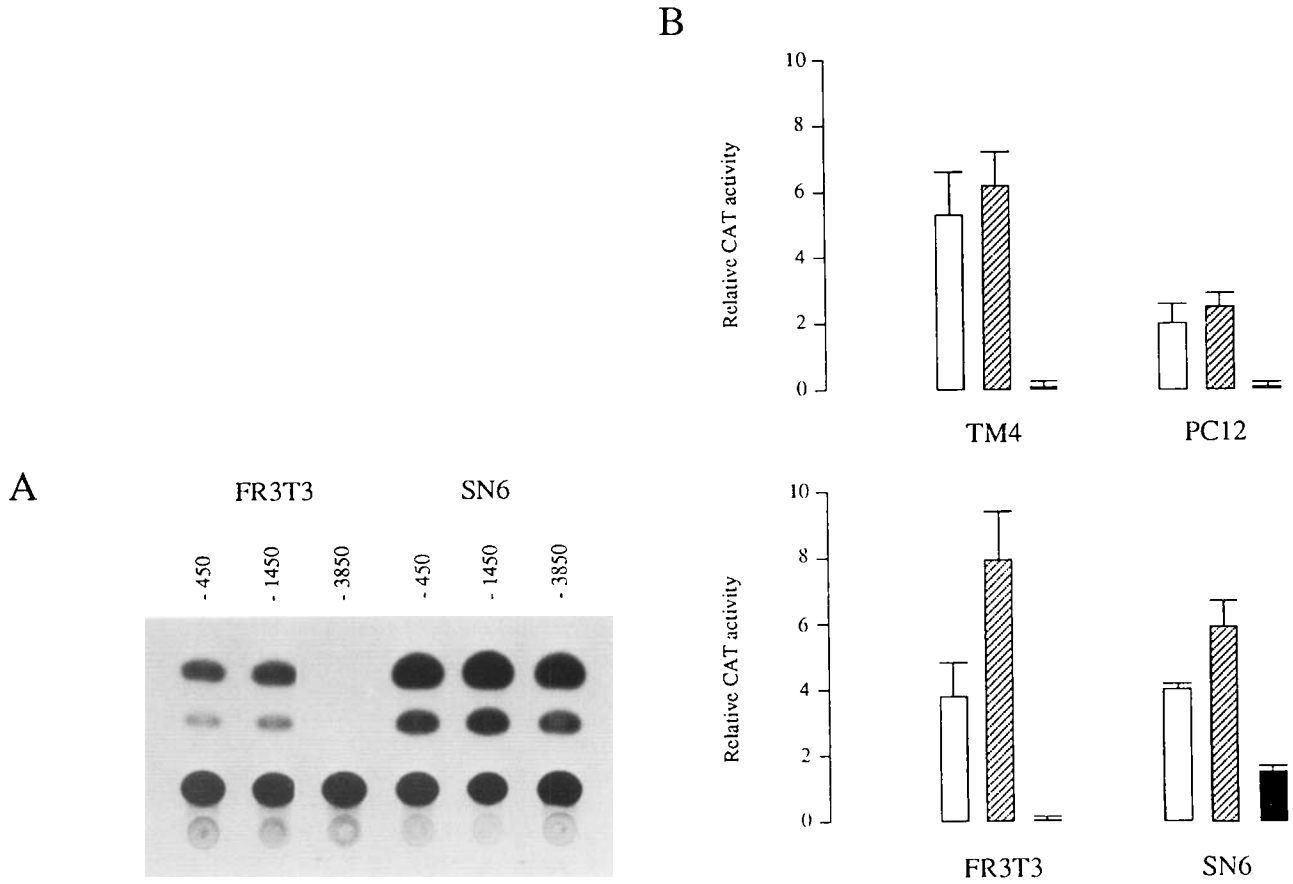


FIG. 4. Activity of the different ChAT promoter constructs in cholinergic and non-cholinergic cell lines. (A) Representative CAT assays from an experiment in which the indicated constructs were transfected into FR3T3 and SN6 cells. Shown in the Figure is an autoradiogram of a prolonged exposure of the TLC plate. Note that, even after a long exposure, no activity from the −3850 construct can be detected in FR3T3 cells. (B) Quantification of ChAT promoter activity in different cell lines. After transfection with the indicated constructs, cell extracts containing equivalent amounts of protein (as measured by absorbance at 280 nm) were assayed for CAT activity. For comparison, CAT activity is expressed here in arbitrary units relative to the activity obtained with the tk promoter, which was set to 10 units in each cell line. CAT activity with the tk promoter in SN6 cells was 4-, 6- and 9-fold higher than in FR3T3, TM4 and PC12 cells, respectively. Open bars, −450 construct; hatched bars, −1450 construct; solid bars, −3850 construct. All results are averages of triplicate transfections ± SD.

the FR3T3 and SN6 cell lines, the −1450 construct was 100 and 50%, respectively, more active than the −450 construct (Fig. 4B). In contrast, the use of the longer −3850 construct allowed CAT activity only in the cholinergic cell line SN6 (although at 2−3-fold lower levels than those seen with the shorter constructs), while CAT activity with this construct was suppressed in the three other cell lines, including PC12 cells (Fig. 4A and B). Using the transcription from the tk promoter as an internal standard, the activities of both the −450 and −1450 constructs were similar in all cell lines except PC12, where the relative levels of CAT activity were 2−4-fold lower than in the other cells (Fig. 4B). Comparisons using the transcription from the SV40 promoter as standard gave similar results (not shown).

### *Proximal sequences are involved in NGF responsiveness of the ChAT promoter*

Cholinergic cells in the septum have been shown to express the low-affinity NGF receptor (NGF-R) (Richardson *et al.*, 1986) and to respond both *in vivo* (Gnahn *et al.*, 1983; Williams *et al.*, 1986; Hefti, 1986; Ernfors *et al.*, 1989) and *in vitro* (Hefti *et al.*, 1985; Martínez, *et al.*, 1987) to NGF. PC12 cells undergo a morphological and biochemical differentiation upon NGF treatment, which includes increased levels of ChAT activity (Schubert *et al.*, 1977; Edgar and Thoenen, 1978). It was therefore of interest to study the effects of NGF on the promoter activity of the different ChAT constructs in both the septum-derived SN6 cells and in PC12 cells. Northern blot analysis showed that the SN6 cells express high levels of NGF-R mRNA (not shown). However, NGF treatment of these cells (up to 500 ng/ml before and/or after transfection) had no effects on the promoter activity of any of the constructs tested, nor did it affect the steady-state level of ChAT mRNA in these cells (not shown).

PC12 cells, on the other hand, clearly started to differentiate after 24 h of NGF treatment. At this time, the cells were transfected and NGF was kept in the medium during and after all transfection steps. In contrast to the lack of response of the SN6 cells, NGF treatment of PC12 cells increased promoter activity ~4-fold from both the −450 and −1450 constructs compared to the levels seen in undifferentiated cells (Fig. 5A and B). Moreover, CAT activity was also detected with the longer −3850 construct (Fig. 5A and B).

## Discussion

In this study, we cloned and analysed the structure of 5′ flanking sequences of the ChAT gene. Functional analysis of these sequences revealed the presence of both positive and negative regulatory elements. Our results indicate that sequences sufficient to promote transcription are present in the first 450 nucleotides of the 5′ flanking region. The fact that the rat ChAT promoter constructs were active in SN6 cells, which derive from the fusion of mouse and human cells, suggests that sequences important for promoter activity in the ChAT gene are conserved among these three species. In FR3T3 and SN6 cells, the −1450 construct was more active than the −450 construct, suggesting that the region between −450 and −1450 contains sequences which can work as enhancer-like elements in these cell lines. The fact that both constructs were active in all four cell lines tested indicates that the first 1450 bp of the 5′ flanking region of the ChAT gene function as a constitutive promoter.

To determine whether sequences conferring cell type specificity were located further upstream, we generated a construct containing an additional 2.4 kb of 5′ flanking DNA. The inclusion of ChAT sequences between −1450 and −3850 suppressed the activity of the

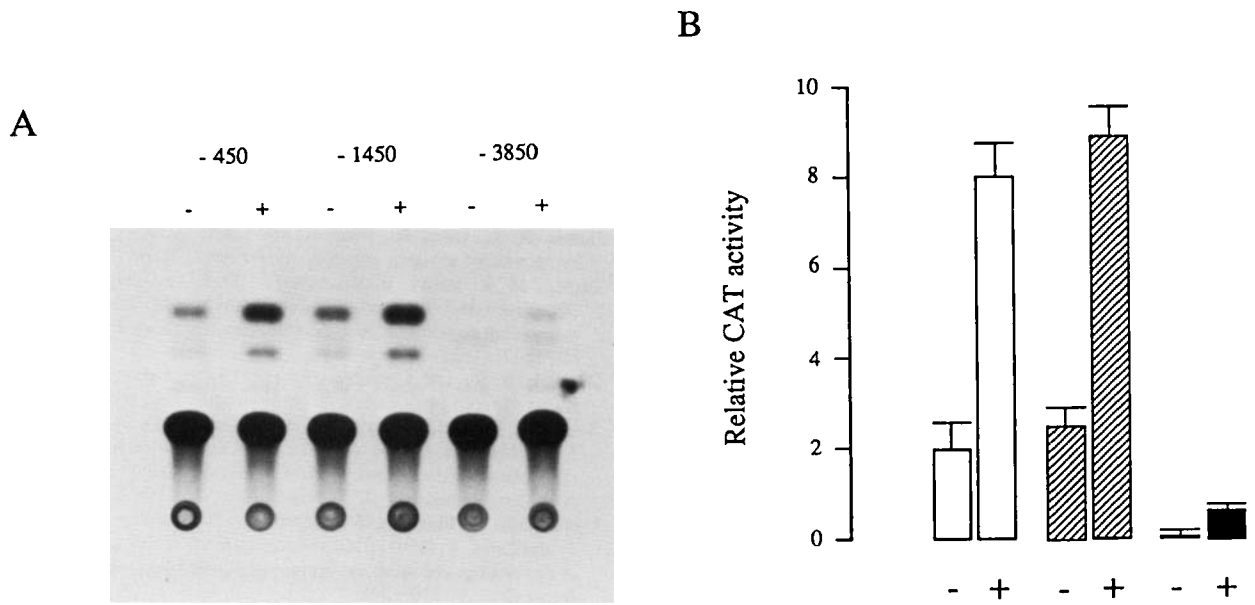


FIG. 5. Effect of NGF on the activity of the different ChAT promoter constructs in PC12 cells. (A) Representative CAT assays from an experiment in which the indicated constructs were transfected into NGF treated (+) or untreated (−) PC12 cells. NGF treatment produces a 4-fold increase in the activity of the −450 and −1450 promoter constructs compared to non-treated cells, and allows detection of low levels of activity from the longer −3850 construct. (B) Quantification of ChAT promoter activity after NGF treatment in PC12 cells. After transfection with the indicated constructs, cell extracts containing equivalent amounts of protein (as measured by absorbance at 280 nm) were assayed for CAT activity. CAT activity is expressed in arbitrary units relative to the activity obtained with the tk promoter, which was set to 10 units in each cell line. Open bars, −450 construct; hatched bars, −1450 construct; solid bars, −3850 construct. All results are averages of triplicate transfections ± SD.

proximal promoter region in fibroblast, Sertoli and phaeochromocytoma cell lines, but did so only 2–3-fold in the cholinergic cell line SN6. This result suggests that the distal region of the ChAT promoter contains a cell type-specific silencer-like element that restricts ChAT gene expression to cholinergic cells. Cholinergic specificity could therefore be achieved through selective derepression by the loss of a silencer-binding factor or the gain of an anti-repressor. Inhibition of transcription has been shown to be important for cell type-specific expression of a number of eukaryotic genes, including the genes for β-interferon (Goodbourn *et al.*, 1986), HSP70 (Williams *et al.*, 1989), c-*myc* (Kakkis *et al.*, 1989) and insulin (Nir and Walker, 1986). In the nervous system, this mechanism has recently been proposed for the neuron-specific expression of neural cell adhesion molecule (NCAM) (Hirsch *et al.*, 1990) and the neural-specific gene SCG10 (Mori *et al.*, 1990).

NGF exerts trophic support of basal forebrain cholinergic neurons and it has been demonstrated both *in vitro* and *in vivo* that this factor induces ChAT activity in these cells. However, NGF treatment of the septum-derived SN6 cells had no effect on the steady-state level of ChAT mRNA, nor did it affect the promoter activity of any of the constructs tested. The lack of response of these cells to NGF could be explained if the fusion from which the SN6 cells originate only allows the low-affinity state of the NGF-R to be expressed. Recently, it has been shown that high-affinity NGF binding requires the coexpression of the product of the low-affinity NGF receptor gene and the product of the proto-oncogene *trk*, which encodes a tyrosine kinase receptor protein (Hempstead *et al.*, 1991). In this context, it is possible that the SN6 cells cannot respond to NGF because they do not express the product of the *trk* gene. Alternatively, the SN6 cells may originate from septal neurons with different trophic requirements, since it has recently been shown that brain-derived neurotrophic factor, which interacts with the low-affinity NGF-R (Rodriguez-Tébar *et al.*, 1990), can promote survival and differentiation of septal cholinergic neurons in culture, and that this effect is additive to the effect of NGF (Alderson *et al.*, 1990; Knusel *et al.*, 1991).

In contrast, a four-fold increase in ChAT promoter activity was seen after NGF-induced differentiation of PC12 cells. This result is in agreement with previous reports that NGF increases ChAT activity in PC12 cells (Schubert *et al.*, 1977; Edgar and Thoenen, 1978), and supports a model in which NGF controls the expression of delayed early genes by activation of regulatory sequences in the 5′ flanking regions of these genes. As demonstrated by the use of the −450 bp construct, sufficient information to allow NGF to increase transcription from the ChAT gene is contained in the first 450 bp of upstream sequence and/or the first 400 bp of transcribed sequence. NGF treatment of PC12 cells also allowed promoter activity from the longer −3850 construct, although at lower relative levels than those seen in SN6 cells, suggesting that this promoter construct still works under partial suppression in differentiated PC12 cells.

It has recently been shown that NGF induction of the promoter for tyrosine hydroxylase, the rate-limiting enzyme for catecholamine synthesis, requires an AP1 site and involves a nucleoprotein complex that contains c-*fos* (Gizang-Ginsberg and Ziff, 1990). Interestingly, in the ChAT gene, seven AP1-like sites are present in 1.5 kb of the 5′ flanking sequence and an additional site is found in the first exon, opening up the possibility that transcription factors of the AP1 complex may also be involved in the mechanism by which NGF induces the ChAT promoter.

The cloning and localization of regulatory and cell type-specific sequences in the ChAT gene promoter reported in this communication will help to unravel the molecular mechanisms by which NGF promotes cell differentiation and to gain insight into the development, maintenance and plasticity of the cholinergic transmitter phenotype in the nervous system.

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## Abbreviations

| | |
|---|---|
| bp | base pairs |
| CAT | chloramphenicol acetyltransferase |
| CDF | choline acetyltransferase developing factor |
| ChAT | choline acetyltransferase |
| CNS | central nervous system |
| EDTA | ethylenediamine tetraacetic acid |
| FCS | fetal calf serum |
| kb | kilobases |
| NCAM | neural cell adhesion molecule |
| NGF | nerve growth factor |
| NGF-R | nerve growth factor receptor |
| PCR | polymerase chain reaction |
| poly($A^+$) RNA | polyadenylated RNA |
| RACE | rapid amplification of cDNA ends |
| SDS | sodium dodecyl sulphate |
| tk | Herpes simplex virus thymidine kinase |
| TLC | thin-layer chromatography |

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