Vesicular GABA transporter and glutamate decarboxylase genes as targets for differentiation of GABAergic neuron: genomic organization and transcriptional regulation

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2002

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SUMMARY

Vesicular GABA transporter (VGAT) and glutamate decarboxylase (GAD) are expressed selectively in GABAergic neurons in invertebrate and mammalian neuron system. Therefore, elucidation of gene expression for these key proteins is essential to understand the mechanism of differentiation of GABAergic neurons. Since VGAT was discovered recently (McIntire *et al*., 1997; Sagne *et al*., 1997), detailed characterization of its gene and expression have not been published. In the present investigation, structure of mouse VGAT (mVGAT) was characterized and analysis of VGAT and GAD expression in cell lines was performed. The mVGAT gene is 4.7 kilobases in size and consisted of three exons and two introns. Analysis of transcripts and genomic DNA identified an alternatively spliced mVGAT isoform (mVGATb). Intron 2 of a previously isolated isoform (mVGATa) was switched over to an exon of mVGATb. In mVGATb, 514 amino acid residues retained in VGATa were flanked by a unique C-terminal sequence of 11 amino acids encoded by intron 2. A major transcription start site of the mVGAT gene was an A residue 209 bp upstream from the translational initiation site.

mVGAT gene was expressed at a high level in retinoic acid (RA)-treated P19 embryonal carcinoma cells, at a very low level in non-treated P19 cells, and not detected in Neuro-2a neuroblastoma cells. The 5'-flanking region exhibited a number of putative regulatory elements including Sp1, Egr-1 and Pitx binding sites. In transient transfection assays, 2 kilobases of the mVGAT 5'-flanking region generated similar levels of luciferase reporter activity in three kinds of cultured cells. Deletion analysis and gel mobility shift assays demonstrated that the region -161 to +155 contained the basal promoter activity of the mVGAT gene and that an activating region from -49 to -27 bound an Sp1-like protein. These arrangements suggest a possible mechanism for regulation of the expression of the mVGAT

gene.

UNC-30, a homeodomain transcription factor in the nematode *C. elegans* specifies the fate of the type D GABAergic neurons, and is required for the expression of *unc*-25/GAD and the vesicular GABA transporter (*unc*-47/VGAT) in the type D neurons (Eastman *et al*., 1999). These observations suggest that Pitx transcription factors, mammalian UNC-30 homologues, are involved in the induction of GABAergic neurons in the mammalian central nervous system.   I analyzed the expression pattern of Pitx2a, Pitx2b and Pitx2c in three kinds of culture cells. These three Pitx2 members were induced to the same extent by RA treatement. Luciferase reporter assay experiments indicate that Pitx2a activate the VGAT and GAD65 promoters. These results suggest that Pitx2a may be associated with the GABAergic neuron differentiation.

In this report, knowledge of the sequence and structure of the mouse VGAT gene will allow us to investigate the function of the VGAT protein by gene targeting experiments followed by analysis of the knockout mice. In addition, further studies of transcriptional regulation of VGAT gene may lead us to clear the molecular mechanisms of GABAergic neuron differentiation in mammals.

Introduction

The differentiation of each neurotransmitter system in each neuron is accompanied by the coordinate expression of the genes involved in the neurotransmitter synthesis and transport (Eiden, 1998; Mallet *et al*., 1998; Goridis and Brunet, 1999; Ernsberger, 2000). Tyrosine hydroxylase and other synthetic enzymes, and vesicular and plasma-membrane monoamine transporters are simultaneously expressed in catecholaminergic neurons (Goridis and Brunet, 1999; Goridis and Rohrer, 2002). Expression of choline acetyltransferase and vesicular acetylcholine transporter genes in cholinergic neuron is regulated cooperatively (Eiden, 1998; Mallet *et al*., 1998). GABAergic neurons also require the expression of a set of genes, including glutamate decarboxylase (GAD) and vesicular GABA transporter (VGAT).

Expression of GABA-related genes has been well investigated in *Caenorhabditis elegans*. In particular, VGAT was discovered from analysis of *unc*-47 mutant (McIntire *et al*., 1993a; McIntire *et al*., 1997; Sagne *et al*., 1997). Based on the sequence of *unc*-47 gene, VGAT cDNA has been cloned from *Caenorhabditis elegans* (McIntire *et al*., 1997), rat (McIntire *et al*., 1997) and mouse (Sagne *et al*., 1997) and the function as VGAT was confirmed (McIntire *et al*., 1997; Sagne *et al*., 1997). VGAT is distributed throughout the central nervous system and, in addition, found in the pancreas and testis at lower levels, indicating a similar expression pattern of VGAT and GAD (McIntire *et al*., 1997). VGAT is detectable at an early embryonic stage and increases during development (Makinae *et al*., 2000). Electrical stimulation of the hippocampus or seizure enhance gene expression of both VGAT (Lamas *et al*., 2001) and GAD67 in rat hippocampus (Schwarzer *et al*., 1995). VGAT activity was competitively inhibited by glycine with low affinity (McIntire *et al*., 1997) and VGAT is expressed not only in GABAergic neurons but also in glycinergic neurons in the rat brain. Therefore, Sagne *et al*. (1997) called VGAT a vesicular amino acid transporter

(VIAAT).

Screening of mutant *Caenorhabditis elegans* genes have identified several other genes that are required for differentiation and function of GABAergic neuron (McIntire *et al*., 1993a,b). Among them is the homeodomain transcription factor UNC-30, which is necessary and sufficient for specifying the fate of type D GABAergic motor neurons that control body locomotion (Jin *et al*., 1994; Hobert *et al*., 1999). UNC-30 is required for the expression of *unc-25/*GAD and the vesicular GABA transporter (*unc-47/*VGAT) in the type D motor neurons (Eastman *et al*., 1999). UNC-30 regulates both of these genes at binding sites in their promoters (Eastman *et al*., 1999). Moreover, ectopic expression of UNC-30 results in the activation of *unc-25/*GAD in non-neural cells (Jin *et al*., 1994). These studies thus identified UNC-30 as an immediate upstream regulator of *unc-25/*GAD and *unc-47/*VGAT, revealing its central role in the specification of the GABAergic neurotransmitter phenotype in *C. elegans*.

Three mammalian transcription factors, Pitx1, Pitx2 and Pitx3, contain homeodomains similar to the UNC-30 homeodomain (Semina *et al*., 1996, 1997; Szeto *et al*., 1996; Smidt *et al*., 1997; Gage *et al*., 1999). Among them, Pitx2 is expressed in several regions of the developing mesencephalon and diencephalon. In embryonic day (E) 11.5 to E12.5 mouse embryos, Pitx2 and the GAD67 gene are expressed in similar patterns within these regions (Mucchielli *et al*., 1996; Kitamura *et al*., 1997; Katarova *et al*., 2000; Smidt *et al*., 2000a, b). In the diencephalon, neural progenitors co-expressing Pitx2 and GAD67 are assembled into zona incerta and thalamic reticular nucleus, which contain a high percentage of GABAergic neurons (Najlerahim *et al*., 1990; Benson *et al*., 1992; Esclapez *et al*., 1993; Mucchielli *et al*., 1996; Katarova *et al*., 2000). In E12.5 mouse, Pitx2 and GABA colocalize in the mesencephalon or future superior coliculus, zona incerta/retromammillary region, and first rhombomere (Martin *et al*., 2002). Furthermore, the expression of Pitx2 is maintained in reticular nuclei of newborn mouse, suggesting its long-lasting effect on these neurons

(Mucchielli *et al*., 1996). In contrast, the CNS expression of Pitx3 is localized to developing dopaminergic neurons in the mesencephalon (Smidt *et al*., 1997), and Pitx1 is expressed outside of the developing CNS (Szeto *et al*., 1996, 1999; Lanctot *et al*., 1999). The results suggest that Pitx2 is a candidate for a regulator of GABAergic differentiation.

GAD and VGAT are essential components of GABAergic neurons. Two GAD isoforms, GAD65 and GAD67, encoded by separate genes, exist in mammalian brain (Behar *et al*., 1994; Bu *et al*., 1992; Erlander *et al*., 1991). Makinae *et al*. (2000) and Yanagawa *et al*. (1997) have previously determined the structure of mouse GAD65 and GAD67 and their promoters in order to investigate the GABAergic neuron-specific gene transcription. However, the gene structure and transcriptional regulation of mouse VGAT is not known. To further elucidate the GABAergic neuron-specific gene expression, I determined the genomic structure of the VGAT gene, analyzed the VGAT promoter activity in cell lines and studied the possible involvement of Pitx2a in GAD and VGAT gene expression in cultured cells.

Materials and Methods

Genomic cloning of the mouse VGAT gene

A primer pair (forward 5'-GGTGAAGTTCTACATCGACGTCAAG-3', reverse 5'-GTGTCCAGTTCATCATGCAGTGGAA-3') was designed from the sequence of mouse VGAT cDNA (Sagne *et al*., 1997). Preliminary experiment confirmed that PCR reactions of mouse brain cDNA or 129SV mouse genomic DNA with these primers produced a single 143-bp product. Then, PCR screening of mouse genomic Bacterial Artificial Chromosome (BAC) library (Genome System Inc., St. Louis, MO) was performed with these primers. Three BAC clones (#44G06, #160L22 and #184J1; Genome System Inc.) were positive, and found to cover the whole gene. These were subcloned into the pBluescriptII KS(-) vector (Stratagene, La Jolla, CA, USA) for further analysis.

DNA Sequencing

DNA was sequenced by automated methods using the BigDye Terminator Cycle Sequencing, FS (Applied Biosystems, Tokyo, Japan), according to the manufacturer's instructions.

Preparation of RNA

Total cellular RNA was prepared from adult mouse brain and cultured cells by standard method (Chomczynski *et al*., 1987). Polyadenylated [poly(A)$^+$] RNA was prepared using Oligotex-dT30 (TaKaRa, Otsu, Japan).

Reverse transcriptase PCR

Total RNA was isolated from mouse brain, RA-treated P19 cells, non-treated P19

cells, or Neuro-2a cells and used as the template for RT-PCR. First, the RNA samples were treated with DNase I using a MessageClean™ kit (GeneHunter Corp., Nashville, TN). After inactivation of the DNase I, cDNA was synthesized using Superscript II RNase H⁻ reverse transcriptase (Gibco BRL) in a reaction mixture containing 5.0 μg of total RNA and a random primer (TaKaRa, Otsu, Japan). One-fifth of the cDNA was then amplified using Ex Taq DNA polymerase (TaKaRa, Otsu, Japan). The sense and antisense oligonucleotide primers for GAD67, VGAT, or hypoxanthine phosphoribosyl transferase (HPRT) are shown in Table 1. Programmable temperature cycling (Perkin-Elmer/Cetus 480, Norwalk, CT) was performed with the following cycle profile: 94°C for 1 min before undergoing 30 cycles of 30 sec at 94°C, 1 min at 55°C and 2 min at 72°C for VGAT; 94°C for 1 min before undergoing 30 cycles of 30 sec at 94°C, 1 min at 58°C and 1 min at 72°C for GAD67; and 94°C for 1 min before undergoing 29 cycles of 1 min at 94°C, 1 min at 62°C and 1 min at 72°C for HPRT. After the last cycle, elongation was extended at 72°C for 7 min. PCR products were run on a 2% agarose gel, and then transferred to a Hybond N⁺ nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, England).

Southern blot analyses were carried out to validate and increase the sensitivity of detection of the PCR products from the VGAT and GAD67 mRNAs. The probes for VGAT and GAD67 were made by labeling Primer-3, Primer-4, Primer-7 and Primer-10, respectively, (Table 1) using a digoxigenin (DIG) oligonucleotide 3'-end labeling kit (Roche Diagnostics, Basel, Switzerland). Nylon membranes were prehybridized in DIG Easy Hyb solution (Roche Diagnostics) at 42°C for 2 hr, and hybridized in DIG Easy Hyb solution containing DIG-labeled GAD67 or VGAT probe at 42°C for 6 h. Membranes were then washed twice in 2 X SSC (1 X SSC is 150 mM NaCl, 15 mM trisodium citrate, pH 7.0)/0.1% SDS (5 min each) at room temperature and washed twice in 0.1 X SSC/0.1% SDS (15 min each) at 50°C. Hybridization was detected using a DIG luminescent detection kit (Roche Diagnostics).

Membranes were incubated in blocking solution containing anti-DIG-alkaline phosphatase (dilution 1:5,000; Roche Diagnostics, Germany) at room temperature for 30 min, followed by incubation of membranes in a detection buffer containing disodium 3-(4-methoxyspiro{1, 2-dioxetane-3, 2'-(5-chloro)tricyclo[3, 3, 1, $1^{3.7}$]decan}-4-yl)phenyl phosphate] (CSPD; dilution 1:100) at room temperature for 5 min. Finally, membranes were exposed to Konica X-ray film (Konica Corp., Tokyo, Japan).

Rapid amplificatoin of cDNA ends (RACE)

5'-RACE and 3'-RACE were performed using a SMART^{TM} RACE cDNA Amplification Kit according to the manufacturer's instructions (Clontech, Palo Alto, CA).

For 3'-RACE, 50 ng of mouse brain poly(A)$^+$ RNA was reverse transcribed using 3'-CDS primer (5'-AAGCAGTGGTAACAACGCAGAGTAC(T)$_{30}$(A/G/C)N-3'). The subsequent PCR amplification was performed using the universal primer mix in the kit (an anchor primer homologous to the 3'-RACE adaptor tail) and an mVGAT-specific primer, 5'-CCAGCAAGAGCGCTCCCCATGCCCTGG-3', which corresponds to nucleotides +4,415 to +4,441 in the genomic sequence of the mouse VGAT gene. For 5'-RACE, cDNA was synthesized from 50 ng of mouse brain poly(A)$^+$ RNA using 5'-CDS primer (5'-(T)$_{25}$(A/G/C)N-3') and SMART II oligonucleotide (5'-AAGCAGTGGTAACAACGCAGAGTACGCGGG-3'). The first primers for subsequent PCR amplifications were the universal primer mix in the kit and an mVGAT-specific primer, 5'-TGGAGCCAGAGGGTGGCAGAGGAGCGC-3', which corresponds to nucleotides +501 to +475, and the nested primers were the nested universal primer in the kit and an mVGAT-specific primer, 5'-CGAAGCCCACCGCTTCCTCATCGGTGGC-3', which corresponds to nucleotides +342 to +315 in the genomic sequence of the mouse VGAT gene.

PCR products were purified using a QIAquick PCR purification kit (Qiagen, Hilden,

Germany) and subcloned into the pGEM-T vector (Promega, Madison, WI, U.S.A.) for DNA sequence analysis.

Cell lines and culture conditions

P19 mouse embryonal carcinoma cells and Neuro-2a mouse neuroblastoma were obtained from the American Type Culture Collection (Rockville, MD). P19 cells were cultured in alpha-Minimal Essential Medium (Gibco BRL) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin. Neuro-2a cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Rockville, MD) supplemented with 10% fetal bovine serum (Gibco BRL), 100 IU/ml penicillin, and 0.1 mg/ml streptomycin. Neuro-2a cells were maintained as monolayers in exponential growth between densities 1 x 10^5 and 1 x 10^6 cells/ml in a tissue culture dish. All cells were maintained at 37°C in a 5% CO_2 atmosphere.

To induce neuronal differentiation, 10^6 P19 cells were cultured in a 10-cm bacterial dish in the presence of 5 μM retinoic acid (RA) (all trans; Sigma, St. Louis, MI) for 2 days (from day 0 to day 2). After aggregation in a bacterial dish, the cells were plated in a tissue culture dish, and were grown further in the presence of RA for 2 days (from day 2 to day 4). Then the cells were grown in the absence of RA for 4 days (from day 4 to day 8) and were harvested for preparation of RNA on day 8. For transfection experiments, luciferase plasmids or Pitx2a expression vector were transfected into the cells on day 6, and the transfected cells were harvested for the luciferase enzyme assay or fixed for cytological analysis on day 8.

Immunocytochemistry

Differentiated P19 cells were grown on collagen-coated glass coverslips. After rinses with phosphate-buffered saline (PBS), the cells were fixed in 2% formalin and 0.25%

glutaraldehyde in 0.1 M PB (pH 7.4) or in 4% paraformaldehyde in 0.1 M PB (pH 7.4) for the use of anti-GABA antibody and that of other antibodies, respectively. After fixation, the cells were treated with cold acetone at -30°C for 5 min or were permealized in PBS containing 0.5% Triton X-100. After blocking with 10% normal goat serum and 0.05% NaN$_3$ in PBS, the cells were incubated with the following primary antibodies: anti-GABA rabbit antiserum (ImmunoNuclear, Stillwater, MN; dilution 1:1,000), anti-GAD67 rabbit antibody (K2; Chemicon, Temecula, CA; dilution 1:1,000), anti-VGAT rabbit antibody (Chemicon; dilution 1:2,500), or anti-neurofilament M rabbit antibody (anti-NF-M: Chemicon; dilution 1:400). All incubations with primary antibody were followed by three rinses in PBS. Specimens were then incubated with 5 mg/ml biotinylated anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) followed by three rinses in PBS. Biotin was detected with streptavidin-Texas Red (Amersham Pharmacia Biotech, Little Chalfont, England; dilution 1:200).

Reporter gene construct and expression plasmid

For promoter reporter assays, I constructed luciferase reporter plasmids under control of various lengths of VGAT genomic DNA fragments. The -1954 construct was made by inserting the 2161-bp of *Eco*RI-*Nco*I fragment into the pGL3-Basic vector (Promega, Madison, WI) upstream of the luciferase gene. The resultant construct was named pGL3-1954 (Fig. 7). The various 5'-deletion constructs were generated by digesting the pGL3-1954 construct with *Nhe*I at pGL3-732 and with another appropriate restriction enzymes (*Bam*HI at pGL3-467, *Apa*I at pGL3-456, *Pst*I at pGL3+2 or *Bss*HI at pGL3+155) (see Fig. 7) followed by the generation of blunt ends and ligation. The pGL3–187, pGL3-161, pGL3-137, pGL3-80, pGL3-59, pGL3-31 and pGL3-13 constructs were generated by the PCR method and then subcloned into the pGL3-Basic vector. For Pitx2a coexpression expreriments, constructs containing subfragments of GAD67 GAD65 and VGAT promoter in front of the luciferase

reporter gene were constructed in the pXP1 vector. The VGAT-1954/+210 and VGAT-467/+210 construct were subcloned with *Eco*RI-*Nco*I and *Bam*HI-*NcoI*, respectively (see Fig. 10B). The GAD65-3508/+550 and GAD67-3334/+112 constructs were subcloned with *Eco*RI-*Nco*I and *Eco*RI-*Sma*I, respectively (see Fig. 10C and D). The GAD65-52/+550 and GAD67-52/+112 constructs were generated by PCR method and then subcloned into the PXP1 vector (see Fig. 10 B and C). The mouse Pitx2a expression vector and (POMC Pitx site)$_3$-Luc construct was kindly provided by Dr Jacques Drouin (Lebel *et al*., 2001).

<u>Transfection and luciferase assay</u>

RA-treated P19 cells, non-treated P19 cells and Neuro-2a cells were transfected by use of LipofectAMINE PLUS Reagent (Gibco BRL). Approximately 2×10^5 cells were plated onto 24-well tissue culture dishes and allowed to grow for 24-72 h (until 50-80% confluent). For VGAT promoter luciferase assay, the cells were co-transfected with 0.4 μg of pGL3-promoter plasmid encoding firefly luciferase and 10 ng of pRL-TK plasmid encoding renilla luciferase (Promega, Madison, WI) used as an internal standard for normalization of the transfection efficiency. For the Pitx2a coexpression experiment, the cells were co-transfected with 0.2 μg of pXP1-promoter plasmid encording firefly luciferase, 0.2 μg of empty or Pitx2a expression vector and 5 ng of CMV-β-Galactosidase vector as an internal control. After 48 hr, the treated cells were washed with PBS and the cell extracts were prepared with 1 x passive lysis buffer (Promega) according to the manufacturer's instructions. Firefly luciferase activities and renilla luciferase activities were measured sequentially using the Dual-Luciferase Reporter Assay System (Promega) and an LB96P luminometer (Berthold, Wildbad, Germany). β-Galactosidase activity was measured using the β-Galactosidase enzyme assay system with reporter lysis buffer (Promega). The ratio of firefly luciferase activity to renilla luciferase or β-Galactosidase activity in each sample served as a measure of normalized

luciferase activity.

Preparation of Nuclear extracts

Approximately 3 x 10^8 non-treated P19 cells were washed with PBS and harvested. The cells were pelleted by centrifugation at 2,000 rpm for 5 min at 4°C. The packed cell volume (PCV) was measured and the cells were resuspended in 5 PCV of hypotonic buffer. (Hypotonic buffer is 10 mM HEPES pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 10 mM KCl.) The cells were pelleted by centrifugation at 2,000 rpm for 5 min at 4°C and resuspended in 4 PCV of hypotonic buffer. The cells were allowed to swell for 10 min on ice, and were broken by 20 strokes with the tight pestle of a Dounce homogenizer (Kontes Glass CO.; > 88% cell breakage). The homogenate was spun at 4,000 rpm for 10 min at 4°C and resuspended in 4 PCV of extraction buffer. (Extraction buffer is 20 mM HEPES pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA, 2 mM EGTA, 2 mM DTT, 25% glycerol.) The extract was rocked on a "Lab-Quake" for 45 min at 4°C and sedimented by centrifugation at 14,000 rpm for 30 min at 4°C. The pellet was redissolved in 1.0 ml of nuclear dialysis buffer (20 mM HEPES ph 7.9, 20% glycerol (vol/vol), 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM DTT), and dialyzed against the same buffer overnight at 4°C. After removal of sediment by centrifugation, the nuclear extracts was quick-frozen in small aliquots and stored at -80°C until use.

Gel mobility shift assay

A probe was made consisting of a double-stranded oligonucleotide corresponding to bp -49 to -27 of the VGAT gene (Figs. 5 and 9A), and was termed Oligo I. Nuclear extracts were preincubated in a 20-ml reaction mixture containing 10 mM Tris-HCl (pH 7.5), 0.5 mM dithiothreitol, 0.5 mM EDTA, 50 mM NaCl, 1 mM MgCl$_2$, 4% glycerol and 1 mg of poly (dI-

dC) at 25°C. After 10 min, approximately 1 x 10^5 cpm of a ^{32}P-end-labeled nucleotide probe was added and the incubation was continued for 20 min. The mixtures, together with 2 ml of loading buffer (50% glycerol, 1 mM EDTA, 0.25% xylene cyanole, and 0.25% bromophenol blue), were electrophoresed in a 4% polyacrylamide gel in 0.5 x TBE buffer (45 mM Tris borate, pH 8.4, 0.1 mM EDTA). For competition assays, a large excess of unlabeled double-strand oligonucleotide competitor was incubated together with the nuclear extract prior to adding the ^{32}P-labeled probe. The oligonucleotides Sp1 (Briggs *et al*., 1986) and NF-Y (Urano *et al*., 1986) used for competition assays were Sp1: 5'-ATTCGATCGGGGCGGGGCGAGC-3'; NF-Y: 5'-CGGTTGGCAGCCAATGAAATACAAAGATGA-3'.

Results

Structure of the mouse VGAT gene

Screening of the mouse genomic BAC library led to the identification and purification of three clones. They yielded PCR products of the expected size when amplified with several VGAT cDNA-specific oligonucleotide primers. The subclones generated from the BAC clones overlapped each other and covered the entire VGAT gene. These subclones were used for complete sequencing of the gene. A complete restriction map of the subclones was prepared using the restriction endonucleases *Bam*HI, *Eco*RI, and *Not*I (Fig. 1A).

The VGAT gene is remarkably compact, spanning only about 4.7 kb. Comparison of this genomic DNA sequence with the cDNA sequence reported by Sagne *et al*. (1997) revealed that the gene is organized in three exons interrupted by two introns (Fig. 1A and B). The translation initiation codon of VGAT is in exon 1. The exon/intron boundaries of the VGAT gene were identified by comparison with the cDNA sequence reported by Sagne *et al*. (1997). All the sequences at the exon/intron boundaries match the eukaryotic 5'-donor and 3'-acceptor consensus splice junction sequence GT-AG (Mount *et al*., 1982; Stephens *et al*., 1992), with the exception of the 3'-acceptor sequence in the 2nd intron (Fig. 1B).

3'-RACE experiments using mouse brain mRNA as a template revealed a polyadenylation signal starting 14 to 15 nucleotides upstream of the poly(A) sequence (Fig. 2A). Nucleotide sequence of VGAT gene was registrated at GenBank nucleotide sequence database (accession No. AB080232).

A unique 387-nt sequence in the mouse VGAT mRNA

A cDNA library from the mouse whole brain was examined by using a forward primer specific for exon 2, nucleotides 5823-5847 (Primer-1 in Fig. 2A, Table 1) and a reverse

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primer complementary to nucleotides 6511-6537 in exon 3 (Primer-2 in Fig. 2A, Table 1). A PCR product of 715 nt was 387 nt larger than expected from 328 nt. Nucleotide sequence of the product is shown in Fig. 2A. mVGAT consisted of that of Sagne *et al*. (1997) and a 387-nt insertion that was contiguous with the exon 3 sequence. Here, mVGATs reported by Sagne *et al*. (1997) and those found in this experiment were termed mVGATa and mVGATb, respectively. The first 514 amino acid residues of the predicted mVGATb protein, starting with the initiator methionine, were identical to those of mVGATa, and were followed by a unique C-terminal sequence of 11 amino acids of mVGATb, encoded by the insertion (Fig. 2B). In the genomic DNA, the 328 nt and the inserted sequence are identical to exonic sequence and intron 2 of mVGATa, respectively.

Expression of these VGAT isoforms in the CNS was studied by RT-PCR and subsequent Southern blot hybridization of mRNA. Figure 3A illustrates the locations of primers and probes as well as the lengths of the expected PCR products. RNA preparations obtained from the cerebrum, cerebellum and brainstem, and spinal cord, yielded a single RT-PCR product (715 bp in size) (Fig. 3B), indicating that the expression of the VGATb was predominant compared with that of VGATa.

Some RT-PCR products in the gel could not be visualized by staining with ethidium bromide. Therefore, Southern blot analysis with enhanced sensitivity was performed with a DIG-labeled VGATa probe. Primer-3 (in Table 1) was specific to VGATa. VGATa product from the CNS regions examined was demonstrated as a major band (328 bp in size) on the Southern blot film, indicating the existence of a VGATa isoform (Fig. 3C). The VGATa level in the cerebellum/brainstem was lower than that of the cerebrum and spinal cord (Fig. 3C). My results suggest that the levels of VGATa mRNA expression are different among each CNS region, and that Sagne *et al*. (1997) isolated VGATa from the brain region rich in VGATa. Primer-4 (in Table 1) specific to the VGATb isoform was used as a VGATb probe. RT-PCR

product from the VGATb isoform was detected uniformly in all CNS regions examined (Fig. 3D). These results confirmed the presence of authentic VGATb mRNA.

5'-Flanking sequences of the mouse VGAT gene

To determine the transcription start site, I analyzed 12 distinct clones derived from 5'-RACE products. Seven clones started at an A residue 209 bp upstream from the translational start site. And this position, numbered +1, was identified as the putative major transcription start site (Fig. 4).

For characterization of the promoter, I determined the nucleotide sequences of the 5'-flanking region from -1,954 to the transcription start site (Fig. 4). This DNA sequence contains a CCAAT box, but lacks a canonical TATA box in the neighborhood of the trasncription start site. This DNA sequence also contains a high G+C content (74% GC content) in the 300 bp preceding the transcription start site. The 5'-flanking region also contained a variety of potential binding sites for transcription factors, including Pitx, Egr-1 and Sp-1.

Expression of mouse VGAT in cultured cells

P19 is a multipotential stem cell line and differentiates into neural cells by treatment with RA (Jones-Villeneuve *et al*., 1982). Lin *et al*. (1996) and Staines *et al*. (1994) have reported the expression of the various neurotransmitter phenotypes of P19 neurons by immunocytochemistry. Among them, the GABAergic phenotype is predomimant, because many neurons express GAD67 and GABA. The neurotransmitter of Neuro-2a cells has not been identified but is not considered GABAergic.

I first investigated expression of VGAT and GAD67 using RT-PCR and Southern hybridization studies (Fig. 5). VGAT mRNA was observed at high levels in mouse brain and

in RA-treated P19 cells, and at a very low level in non-treated P19 cells, but was not detected in Neuro-2a cells (Fig. 5A and B).

GAD67 transcripts undergo alternative splicing during the development of the CNS (Szabo *et al*., 1994). The alternatively spliced variant, GAD67E, which includes exon F, is preferentially expressed in the fetal brain (Szabo *et al*., 1994; Yanagawa *et al*., 1997). In contrast, an adult form of GAD67 mRNA, GAD67A, does not contain exon F. PCR primers were designed to amplify either a 267-bp product or a 187-bp GAD67 product, which contain an exon F insert or not, respectively. Furthermore, a probe homologous to a region in both the large and the small amplification products permitted the detection of both PCR products. In total, GAD67 mRNA was expressed at high levels in the mouse brain and in RA-treated P19 cells and at a very low level in non-treated P19 cells (Fig. 5C and D). The expression was not detected in Neuro-2a cells. My results are consistent with the finding of Bain *et al*. (1993) that non-treated P19 cells expressed the GAD67 gene at a very low level and that RA-treated P19 cells expressed the GAD67 gene at high levels. GAD67E was predominantly expressed in non-treated P19 cells and in RA-treated P19 cells, whereas GAD67A was predominant in the mouse brain, as expected (Fig. 5C and D). HPRT was detected in all four RNA samples at approximately equal intensity (Fig. 5E).

Neuronal differentiation of the P19 cells after RA treatment was also confirmed histologically (Fig. 6A and B) and by immunocytochemistry (Fig. 6C). In agreement with the previous findings (Lin *et al*., 1996; Staines *et al*., 1994), a significant number of neurons expressed GAD67 and contained GABA in RA-treated P19 cells (Fig. 6D and E). VGAT and GAD genes are coexpressed in the GABAergic neurons in the CNS of both rats and mice (McIntire *et al*., 1997; Sagne *et al*., 1997). I also detected VGAT gene expression in P19 neurons using immunocytochemistry (Fig. 6F).

Analysis of the promoter activity of the mouse VGAT gene

Promoter activity of the 5'-flanking region of the VGAT gene was assayed by transfection of cell lines with reporter plasmids. I transfected RA-treated P19 cells with the luciferase-encoding plasmid pGL3-1954, in which bp -1954 to +155 of the mouse VGAT gene was linked to the luciferase reporter gene. The promoterless pGL3-Basic and the pGL3-Promoter driven by the SV40 early promoter were also transfected into RA-treated P19 cells. In RA-treated P19 cells, which originally expressed VGAT, the luciferase activity after pGL3-1954 transfection was 6-fold higher than that generated by pGL3-Basic, and was 49% of that generated by pGL3-Promoter (Fig. 7). These data indicate that the 5'-flanking region of VGAT contains moderate promoter activity. To further localize the *cis*-acting elements, I generated reporter plasmids with various lengths of the VGAT 5'-flanking region and transfected these plasmids into RA-treated P19 cells. As few as 161 bp of the VGAT promoter directed a high level of promoter activity; however, deletion of another 130 bp of the promoter sequence (pGL3-31, Fig. 7) decreased the luciferase activity markedly. These data indicate that nt -161 to -31 of the VGAT gene contain most of the promoter activity.

To determine whether about 2 kbp of the VGAT 5'-flanking sequence contained GABAergic neuron-specific element(s), I transfected luciferase plasmids into two different cell types in culture, non-treated P19 cells and Neuro-2a cells. As shown in Fig. 5, non-treated P19 cells express VGAT at very low levels, and Neuro-2a cells do not express VGAT. Both cells showed distinctive activity and the relative activity with pGL3-1954 (measured using pGL3-Promoter as reference) was 42% in non-treated P19 cells and 68% in Neuro-2a cells. Changes in luciferase activity by successive deletion of pGL3-1954 to pGL3+155 in non-treated P19 cells (Fig. 7) and in Neuro-2a cells (data not shown) were similar in their pattern to those in RA-treated P19 cells, although the activities of pGL3-467 to -80 were apparently higher in RA-treated P19 cells than in non-treated ones. Thus, VGAT 5'-flanking region

stimulated VGAT gene transcription not only in RA-treated P19 cells but also in non-treated P19 cells and in Neuro-2a cells. These data raise the posssibility that GABAergic neuron-specific elements are located in other regions upstream of -1954 bp, the introns, the 3' region of the gene or were undetectable because of a small proportion of GABAergically differentiated neurons in culture. The region from -161 to +155 functioned as a promoter with activity similar to or even greater than the SV40 promoter, not only in RA-treated P19 cells but also in non-treated P19 cells and in Neuro-2a cells. The progressive removal of the 5'-sequences from -161 to -31 resulted in a drastic decrease of the luciferase activity. These results suggest that positive *cis*-acting regulatory elements exist in the region from -161 to -31 in all cell types examined and that this region has basal mVGAT promoter activity.

Investigation of nuclear protein bound to promoter region

Since the region from -59 to -31 bp of the mVGAT gene possessed a basal activating function (Fig. 7), a gel mobility shift assay using nuclear extracts from non-treated P19 cells was performed to find any specific binding protein. In gel mobility shift assays, I searched a specific DNA-protein complex using Oligo I consisting of bp -49 to -27 (which contains a possible Sp1 binding site; Fig. 8A) as a probe and found a single DNA-protein complex (Fig. 8B). This complex was a specific as a 200-fold molar excess of unlabeled Oligo I competitor (Fig. 8B, lane 6) abolished the formation of the binding complex, but a 2,000-fold excess of unrelated oligonucleotide NF-Y did not (Fig. 8B, lane 8). In addition, a 200-fold molar excess of unlabeled consensus Sp1 oligonucleotide reduced the formation of the DNA-protein complex (Fig. 8B, lane 3). These results suggest that Sp1-like protein(s) may be present in the DNA-protein complex and that the binding of the Sp1-like protein to -49/-27 must be stronger than to the Sp1 consensus sequence. The identification of this protein is a matter of further investigation.

Expression of mouse Pitx2a, Pitx2b and Pitx2c in cultured cells

Pitx transcription factors Pitx2a, Pitx2b and Pitx2c in mammals contain homeodomains similar to the UNC-30 homeodomain (Semina *et al*., 1996). They are generated from a single Pitx2 gene by alternative splicing (Kitamura *et al*., 1999). Westmoreland *et al*. (2001) have found that Pitx2 rescues the defect in GABAergic differentiation and behavioral phenotype of *unc*-30 mutants. However, it remains unclear whether or not Pitx transcription factors are involved in the induction of GABAergic neurons in the mammalian central nervous system.

Expression of Pitx2a, Pitx2b and Pitx2c were examined in mouse brain and cultured cells using RT-PCR analysis (Fig. 9). All three Pitx2 members were expressed in the mouse brain, although the expression of Pitx2a appeared highest. Transcripts of Pitx2 were expressed at high or moderate levels in RA-treated P19 cells, and at moderate or low levels in non-treated P19 cells, but they were not expressed in Neuro-2a cells. The enhanced expression of Pitx transcription factors, in differentiated P19 cells was similar to that of GABAergic genes, GAD67 and VGAT (Fig. 5). These results suggest that Pitx transcription factors are associated with the expression of GABAergic genes.

Pitx2a activates the mouse VGAT and GAD65 promoters

In order to determine if Pitx2a activates the VGAT, GAD65 and GAD67 promoters, these promoters were linked to a luciferase vector, pXP1 (pVGAT-1954/+210, pVGAT-467/+210, pGAD65-3508/+550, pGAD65-52/+550, pGAD67-3334/+112 and pGAD67-52/+112) and cotransfected into RA-treated P19 cells with the expression plasmid, pRSV-Pitx2a or control pRSV vector (Fig. 10). POMC promoter-luciferase construct was used as a positive control (Lebel M. *et al*., 2001). Pitx2a activated the POMC promoter three-fold (Fig. 10A), and these results are consistent with those reported by Lebel *et al*. (2001).

Cotransfection of pRSV-Pitx2a resulted in a two- to three-fold increase in the luciferase activities of promoter constructs pVGAT-1954/+210, pVGAT-467/+210, pGAD65-3508/+550, and pGAD65-52/+550 (Fig. 10B and C). The activities were higher in the shorter constructs which lack the Pitx element. These results suggest that Pitx2a activates VGAT and GAD65 promoters through other unidentified elements. However, cotransfection of pRSV-Pitx2a did not affect the luciferase activities of constructs pGAD67-3334/+112 or pGAD67-52/+112. These results suggest that these GAD67 promoter might not contain a Pitx2a responsive element.

Discussion

In the first part of the present investigation, I cloned and sequenced 6,668 bp of genomic DNA for mouse VGAT (mVGAT), and thus identified exon/intron boundaries, transcription start sites and polyadenylation signals as well as regulatory regions that confer basal promoter activity.

First, I defined three exons with characteristic exon/intron boundaries. The cording region of the mVGAT gene is spread over 4.7 kb. The cDNAs derived from the rat and mouse VGAT genes predicts a protein product containing 10 transmembrane domains and a large hydrophilic N-terminal domain (McIntire *et al.*, 1997; Sagne *et al.*, 1997). The N-terminus and C-terminus are assumed to be cytosolic because of the lack of a signal peptide. The structure of the VGAT gene indicated that exon 1 may form a large hydrophilic N-terminal domain, exon 2 may include all of the transmembrane domains, and exon 3 may correspond to the C-terminal domain.

I also identified a novel isoform of VGAT mRNA, named VGATb, in addition to that, of Sagne *et al.* (1997) named VGATa. Both VGAT mRNAs might be produced by alternative splicing of a single VGAT gene in the mouse brain. Sequencing of the genomic DNA and identification of splice sites substantiated the alternative splicing event. Different amino acid residues were predicted in the carboxyl tails of the mVGAT isoforms. Carboxyl tails in the rat VGAT (rVGAT) and human VGAT (hVGAT) (GenBank accession No. AK055051) were differ from mVGATa proteins (McIntire *et al.*, 1997; Sagne *et al.*, 1997). I compared the carboxy-terminus 11 amino acid residues of mVGATb with those of rVGAT (McIntire *et al.*, 1997) or hVGAT, and found that the carboxy-terminus amino acid residues of mVGATb are identical with those of rVGAT or hVGAT. Both 5'-donor site and 3'-acceptor site of the mouse VGAT intron 2 did not conserve in rat and human. These results suggest the

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possibility that mVGATa form is only in mouse exceptionally. Both rVGAT and mVGATa exert vesicular GABA transport activity using transfected cells with VGAT cDNA (McIntire *et al*., 1997; Sagne *et al*., 1997), suggesting that the different carboxy-terminal amino acid residues of mVGATa and mVGATb do not affect the intrinsic transport mechanism. The alternatively spliced isoform VGATb is predominantly expressed in several rat brain regions (McIntire *et al*., 1997), although my data could not determine the precise quantitative ratio of VGATb to VGATa. I searched a functional motif in the different carboxy-terminus of mVGAT using the PROSITE Pattern database (Hofmann *et al*., 1999) and did not find the known functional protein motif. Finally, it is not known whether these splice variants have distinct physiological functions and are therefore under different regulation of expression in the CNS.

In the second part of the present investigation, I analyzed the expression of VGAT, GAD65 and GAD67 in cell lines and the possible roles of Pitx2 transcripion factor. RA treatment induces expression of a variety of neuronal markers and morphological characteristics in P19 embryonal carcinoma cell line (McBurney *et al*., 1993). When studied with Northern analysis, GAD65 and GAD67 are expressed in the P19 neurons indicating that a population of P19 neurons are GABAergic (Bain *et al*., 1993). In addition, Lin *et al*. (1996) observed spontaneous electrical activity in differentiated P19 neurons and the blockade of this activity by a GABA antagonist, bicuculline. These findings suggest that P19 neurons not only synthesize GABA but also release GABA, which exerts excitatory action as commonly observed in premature neurons. Then, I conducted functional analyses of the mouse VGAT promoter in P19 cells. RT-PCR experiments revealed expression of VGAT gene, and immunocytochemistry demonstrated the existence of VGAT and GAD67 in RA-treated P19 neurons. Therefore, RA-treated P19 cells, are useful as an *in vitro* model of GABAergic neurons.

I determined the nucleotide sequence of the 5'-flanking region of the mVGAT gene.

Absence of a TATA box in this sequence predicts the presence of multiple transcription start sites, since many genes with TATA-less promoters contain multiple transcription start sites (Geng *et al.*, 1993). Inspection of the sequence further revealed consensus binding motifs for transcription factors Sp1, Egr-1 and Pitx. The 5'-flanking regions of the VGAT and human VGAT genes (GenBank accession No. AL133519) have high homology, suggesting the similar regulatory mechanism for the expression of this gene. The nucleotide sequences of the Sp1 and Egr-1 regulatory elements in the mouse and human VGAT promoters were almost identical. We previously determined a nucleotide sequence of more than 4 kb of the promoter region and exon 1 of the mouse GAD65 gene (Makinae *et al.*, 2000) and that of more than 10 kb of the promoter region and upstream exons of the mouse GAD67 gene (T. Kobayashi *et al.*, unpublished data), but I found no significant homology among the 5'-flanking regions or the promoters of the mVGAT, GAD65 and GAD67 genes.

I tested the transcriptional activity of the mVGAT promoter in cultured cell lines. The relative promoter activity of all mVGAT constructs in RA-treated P19 cells did not differ significantly from that in non-treated P19 cells or Neuro-2a cells. These findings suggest that regulatory elements mediating GABAergic neuron-specific expression are absent at least in the 1.9 kb upstream of the transcription start site and are presumably located outside the non-selective promoter or that the percentage of GABA-positive cells in RA-treated P19 cells was so small (3% of total cells) that GABAergic neuron-specific expression could not detected.

I generated the successively deleted mVGAT promoter fragments, and examined their transcriptional activities. pGL3-161 showed the highest activity, which was even higher than that of the viral SV40 promoter in pGL3-Promoter. PGL3-161 was similarly high in transcriptional activation not only in RA-treated P19 cells but also in non-treated P19 cells or in Neuro-2a cells, suggesting that the –161 region of VGAT may contain a basal promoter activity. Further deletion analysis demonstrated that ablation of the Sp1 motifs led to loss of

basal promoter activity. These results raise the possibility that the presence of the Sp1 motifs is necessary for mVGAT promoter activity. Competitive mobility shift studies for the region from nt -59 to -31 showed that the VGAT gene-protein complex was partially suppressed by the Sp1 consensus oligonucleotide. Sp1 is important for enhancing the expression of some TATA-less genes (Emami *et al*., 1998). The nuclear protein(s) I detected by gel shift assay might belong to the family of Sp1 transcription factors and play a major role in the basal promoter activity. Sp1-like proteins such as Sp2, Sp3 or Sp4 are possible candidates (Chen *et al*., 1998; Geng *et al*., 1999; Scohy *et al*., 1998), but without further cloning I cannot ascertain the molecular identity of the binding protein.

As described in the Introduction, Pitx2 is suggested to be involved in differentiation of GABAergic neurons. This possibility was evaluated in cultured cells. RA-induced neuronal differentiation of P19 cells resulted in an increase of the expression of Pitx2, GAD67 and VGAT. Three Pitx2 members, Pix2a, Pix2b and Pix2c were induced to the same extent. Pitx2a activates the VGAT and GAD65 promoters in RA-treated P19 cells. These results suggest the possibility that Pitx2a induces the GABAergic neuron differentiation in RA-treated P19 cells. On the other hand, Pitx2a did not activate the GAD67 promoter, although activated the VGAT and GAD65 promoter in RA-treated P19 cells. A possible explanation for the different activations is that elements required for Pitx2 activation are not present within the 3.3 kb fragment of the mGAD67 gene. Thus, it is possible that additional regulatory elements are embedded in sequences further upstream or within intragenic sequences or in the 3'-flanking sequences.

Pitx2 may indirectly activate GABAergic genes, because transfection with generated curtailed VGAT and GAD65 promoter fragments showed that removal of the putative Pitx binding motif did not affect the promoter activity (Fig. 10). Green *et al*. (2001) reported that Pitx2 regulates Dlx2 gene expression. Dlx2 is a transcription factor, which is a

member of the distal-less gene family and is expressed in the diencephalon and in the mandibular and maxillary regions. Pitx2 and Dlx2 genes are expressed in the same tissues during early development. Ectopic expression of Dlx2 in slice cultures of the mouse embryonic cerebral cortex induced the expression of GAD65 and GAD67 (Stuhmer *et al.*, 2002). Taken together, it is possible that Pitx2 activates the expression of GABAergic genes via activation of Dlx2. It is still possible, however, that Pitx2 may directly activate the expression of GABAergic genes.

Here I characterized the structural organization of the mVGAT gene as well as the structure and function of its promoter. Characterization of the exon-intron boundaries and of the introns and knowledge of the chromosomal position of the VGAT gene will be useful to analyze its mutation in various diseases which are ascribed to defects in GABA function. Precise structure of the mVGAT gene will allow gene targeting of VGAT and production of VGAT knockout mice. GAD65 and GAD67 knockout mice have been reported (Asada *et al.*, 1996; Asada *et al.*, 1997; Ji *et al.*, 1999; Stork *et al.*, 2000). GAD65 knockout mice show susceptibility to seizures and abnormal emotional behavior, whereas GAD67 knockout mice die of cleft palate. The roles of the VGAT protein in the function of GABA will be investigated by analysis of VGAT knockout mice and their comparison with that of GAD65 or GAD67 knockout mice.

Acknowledgements

First of all, I would like to give my best thanks to Professor K.Obata for coutinuous encouragement and fruitful discussion. Dr. Y. Yanagawa kindly taught me the general techniques for molecular biology and useful suggestions. I am grateful to Dr. S. Furuya for immunohistochemistry and *in situ* hybridization techniques and to Dr. K. Kaneko and Dr. Y. Yamagata for useful discussions. I thank K. Makinae for technical help and useful discussions. I am grateful to Dr. J. Drouin for providing the Pitx2 expression vector. I am thankful to Dr. T. Suzuki, Dr. H. Mizusaki, Dr T. Ikeuchi and Mr. T. Notomi for critical discussion. I am grateful to Ms. M. Owada for her secretarial assistance. Finally, I would deeply appreciate the cooperation of all the members of the National Institute for Physiological Sciences.

References

1. Asada H, Kawamura Y, Maruyama K, Kume H, Ding R, Ji F.Y., Kanbara N, Kuzume H, Sanbo M, Yagi T, Obata K. (1996). Mice lacking the 65 kDa isoform of glutamic acid decarboxylase (GAD65) maintain normal levels of GAD67 and GABA in their brains but are susceptible to seizures, Biochem. Biophys. Res. Commun. 229, 891-895.

2. Asada H, Kawamura Y, Maruyama K, Kume H, Ding R.G, Kanbara N, Kuzume H, Sanbo M, Yagi T, Obata K. (1997). Cleft palate and decreased brain gamma-aminobutyric acid in mice lacking the 67-kDa isoform of glutamic acid decarboxylase, Proc. Natl. Acad. Sci. USA 94, 6496-6499.

3. Bain G., Ramkumar T.P., Cheng J.M., Gottlieb D.I. (1993). Expression of the genes coding for glutamic acid decarboxylase in pluripotent cell lines, Brain. Res. Mol. Brain Res.   17, 23-30.

4. Behar T., Ma W., Hudson L., Barker J.L. (1994). Analysis of the anatomical distribution of GAD67 mRNA encoding truncated glutamic acid decarboxylase proteins in the embryonic rat brain, Brain Res. Dev. Brain Res. 14, 77-87.

5. Benson D.L., Isackson P.J., Gall C.M., Jones E.G. (1992). Contrasting patterns in the localization of glutamic acid decarboxylase and Ca2+/calmodulin protein kinase gene expression in the rat central nervous system, Neuroscience 46, 825-849

6. Briggs M.R., Kadonaga J.T., Bell S.P., Tjian R.(1986). Purification and biochemical characterization of the promoter-specific transcription factor, Sp1, Science 234, 47-52.

7. Bu D. -F, Erlander M. G., Hitz B. C., Tillakaratne N. J. F., Kaufman D. L., Wagner McPherson C. B., Enans G. A., Tobin A.J. (1992). Two human glutamate decarboxylases, 65-kDa GAD and 67-kDa GAD, are each encoded by a single gene, Proc. Natl. Acad. Sci. USA 89, 2115-2119

8. Chen S.J., Artlett C.M., Jimenez S.A., Varga J. (1998). Modulation of human alpha1(I)

procollagen gene activity by interaction with Sp1 and Sp3 transcription factors *in vitro*, Gene 215, 101-110.

9. Chomczynski P., Sacchi N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, Anal. Biochem. 162, 156-159.

10. Eastman C, Horvitz H.R., Jin Y. (1999). Coordinated transcriptional regulation of the *unc-25* glutamic acid decarboxylase and the unc-47 GABA vesicular transporter by the Caenorhabditis elegans UNC-30 homeodomain protein, J. Neurosci. 19, 6225-6234.

11. Eiden LE (1998) The cholinergic gene locus. J Neurochem 70, 2227-2240

12. Emami K.H., Burke T.W., Smale S.T. (1998). Sp1 activation of a TATA-less promoter requires a species-specific interaction involving transcription factor IID, Nucleic. Acids Res. 26, 839-846.

13. Erlander M.G., Tillakaratne N.J.K., Feldblum S., Patel N., Tobin A.J. (1991). Two genes encode distinct glutamate decarboxylases, Neuron 7, 91-100.

14. Ernsberger U. (2000). Evidence for an evolutionary conserved role of bone morphogenetic protein growth factors and phox2 transcription factors during noradrenergic differentiation of sympathetic neurons. Induction of a putative synexpression group of neurotransmitter-synthesizing enzymes. Eur J Biochem 267, 6976-6981.

15. Esclapez M., Tillakaratne N.J.K., Tobin A.J., Houser C.R. (1993). Comparative localization of mRNAs encoding two forms of glutamic acid decarboxylase with nonradioactive *in situ* hybridization methods, J Comp Neurol 331, 339-362.

16. Gage P.J., Suh H., Camper S.A., (1999). The bicoid-related *Pitx* gene family in development, Mamm Genome 10, 197-200

17. Goridis C., Brunet JF. (1999). Transcriptional control of neurotransmitter phenotype. Curr Opin Neurobiol 9, 47-53.

18. Goridis C, Rohrer H. (2002) Specification of catecholaminergic and serotonergic neurons.

Nat Rev Neurosci. 3, 531-541.

19. Geng Y,, L.F. Johnson, Lack of an initiator element is responsible for multiple transcriptional initiation sites of the TATA-less mouse thymidylate synthase promoter, Mol. Cell.Biol. 13 (1993) 4894-4903.

20. Geng Y., Tsai-Morris C.H., Zhang Y., Dufau M.L. (1999). The human luteinizing hormone receptor gene promoter: activation by Sp1 and Sp3 and inhibitory regulation, Biochem. Biophys. Res. Commun. 263, 366-371.

21. Green P.D., Hjalt T.A., Kirk D.E., Sutherland L.B., Thomas B.L., Sharpe P.T., Snead M.L., Murray J.C., Russo A.F., Amendt B.A. (2001). Antagonistic regulation of Dlx2 expression by PITX2 and Msx2: implications for tooth development, Gene Expr. 9, 265-281.

22. Hobert O, Tessmar K., Ruvkun G. (1999). The Caenorhabditis elegans lim-6 LIM homeobox gene regulates neurite outgrowth and function of particular GABAergic neurons, Development 126, 1547-1562.

23. Hofmann K., Bucher P., Falquet L., Bairoch A. (1999). The PROSITE database, its status in 1999, Nucleic. Acids. Res 27, 215-219

24. Ji F., Kanbara N., Obata K. (1999). GABA and histogenesis in f*et al* and neonatal mouse brain lacking both the isoforms of glutamic acid decarboxylase, Neurosci. Res. 33, 187-194.

25. Jin Y, Hoskins R., Horvitz H.R. (1994). Control of type-D GABAergic neuron differentiation by C. elegans UNC-30 homeodomain protein, Nature 372 780-783.

26. Jones-Villeneuve E.M., McBurney M.W., Rogers K.A., Kalnins V.I. (1982). Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells, J. Cell. Biol. 94, 253-262.

27. Katarova Z., Szabo G., Mugnaini E., Greenspan R.J. (1990). Molecular identification of the 62 kd form of glutamic acid decarboxylase from the mouse, Eur. J. Neurosci. 2, 190-202.

28. Katarova Z., Sekerkova G., Prodan S., Mugnaini E., Szabo G. (2000). Domain-restricted

expression of two glutamic acid decarboxylase genes in midgestation mouse embryos, J Comp Neurol 424, 607-627

29. Kitamura K., Miura H., Yanazawa M., Miyashita T., Kato K. (1997). Expression patterns of *Brx1* (*Rieg* gene), *Sonic hedgehog*, *Nkx2.2*, *Dlx1* and *Arx* during zona limitans intrathalamica and embryonic ventral lateral geniculate nuclear formation, Mech Dev 67, 83-96

30. Kitamura K., Miura H., Miyagawa-Tomita S., Yanazawa M., Katoh-Fukui Y., Suzuki R., Ohuchi H., Suehiro A., Motegi Y., Nakahara Y., Kondo S., Yokoyama M. (1999). Mouse Pitx2 deficiency leads to anomalies of the ventral body wall, heart, extra- and periocular mesoderm and right pulmonary isomerism.Development. 126, 5749-5758.

31. Konecki D.S., Brennand J., Fuscoe J.C., Caskey C.T., Chinault A.C. (1982). Hypoxanthine-guanine phosphoribosyltransferase genes of mouse and Chinese hamster: construction and sequence analysis of cDNA recombinants, Nucleic. Acids Res. 10, 6763-6775.

32. Lamas M., Gomez-Lira G., Gutierrez R. (2001). Vesicular GABA transporter mRNA expression in the dentate gyrus and in mossy fiber synaptosomes, Brain Res. Mol. Brain Res. 93, 209-214.

33. Lanctot C., Gauthier Y., Drouin J. (1999). Pituitary homeobox 1 (*Ptx1*) is differentially expressed during pituitary development, Endocrinology 140. 1416-1422.

34. Lebel M, Gauthier Y, Moreau A, Drouin J. (2001). Pitx3 activates mouse tyrosine hydroxylase promoter via a high-affinity binding site, J Neurochem. 77, 558-567.

35. Lin P., Kusano K., Zhang Q., Felder C.C., Geiger P.M., Mahan L.C. (1996). GABAA receptors modulate early spontaneous excitatory activity in differentiating P19 neurons, J. Neurochem. 66, 233-242.

36. Martin D.M., Skidmore J.M., Fox S.E., Gage P.J., Camper S.A.(2002). Pitx2 distinguishes

subtypes of terminally differentiated neurons in the developing mouse neuroepithelium, Dev.Biol. 252, 84-99

37. Makinae K., Obata K., Yanagawa Y. (2000). Developmental expression of vesicular GABA transporter in mouse brain, Soc. Neurosci. Abst. 26, 17.

38. Makinae K., Kobayashi T., Kobayashi T., Shinkawa H., Sakagami H., Kondo H., Tashiro F., Miyazaki J., Obata K., Tamura S., Yanagawa Y. (2000). Structure of the mouse glutamate decarboxylase 65 gene and its promoter: preferential expression of its promoter in the GABAergic neurons of transgenic mice, J. Neurochem. 75, 1429-1437.

39. Mallet J., Houhou L., Pajak F., Oda Y., Cervini R., Bejanin S., Berrard S. (1998). The cholinergic locus: *ChAT* and *VAChT* genes. J Physiol (Paris) 92, 145-147

40. McBurney M.W. (1993). P19 embryonal carcinoma cells, Int. J. Dev. Biol. 37, 135-140.

41. McIntire S.L., Jorgensen E., Horvitz H.R.(1993a). Genes required for GABA function in Caenorhabditis elegans, Nature 364, 334-337.

42. McIntire S.L, Jorgensen E, Kaplan J, Horvitz H.R.(1993b). The GABAergic nervous system of *Caenorhabditis elegans*, Nature 364, 337-341

43. McIntire S.L., Reimer R.J., Schuske K., Edwards R.H., Jorgensen E.M.(1997). Identification and characterization of the vesicular GABA transporter, Nature 389, 870-876.

44. Mount S.M. (1982). A catalogue of splice junction sequences, Nucleic. Acids Res. 10, 459-472.

45. Mucchielli M.L., Martinez S., Pattyn A., Goridis C., Brunet J-F. (1996). *Otlx2*, an *Otx*-related homeobox gene expressed in the pituitary gland and in a restricted pattern in the forebrain, Mol Cell Neurosci 8, 258-271.

46. Mucchielli M.L., Martinez S., Pattyn A., Goridis C., Brunet J-F. (1996). *Otlx2*, an *Otx*-related homeobox gene expressed in the pituitary gland and in a restricted pattern in the forebrain, Mol Cell Neurosci 8, 258-27.

47. Najlerahim A., Harrison P.J., Barton A.J.L., Heffernan J., Pearson R.C.A. (1990). Distribution of messenger RNAs encoding the enzymes glutaminase, aspartate aminotransferase and glutamic acid decarboxylase in rat brain, Mol Brain Res 7, 317-333

48. Sagne C.,Mestikawy S. El., Isambert M.F., Hamon M., Henry J.P., Giros B., Gasnier B. (1997). Cloning of a functional vesicular GABA and glycine transporter by screening of genome databases, FEBS Lett. 417, 177-183.

49. Semina E.V., Reiter R., Leysens N.J., Alward W.L., Small K.W., Datson N.A., Siegel-Bartelt J., Bierke-Nelson D., Bitoun P., Zabel BU., Carey JC., Murray JC. (1996). Cloning and characterization of a novel bicoid-related homeobox transcription factor gene, RIEG, involved in Rieger syndrome, Nat Genet.14, 392-399.

50. Semina E.V., Reiter R.S., Murray J.C., (1997). Isolation of a new homeobox gene belonging to the *Pitx/Rieg* family: expression during lens development and mapping to the *aphakia* region on mouse chromosome 19, Hum Mol Genet 6, 2109-2116.

51. Schwarzer C, Sperk G. (1995). Hippocampal granule cells express glutamic acid decarboxylase-67 after limbic seizures in the rat, Neuroscience. 69, 705-709.

52. Scohy S., Van Vooren P., Szpirer C., Szpirer J. (1998). Assignment1 of Sp genes to rat chromosome bands 7q36 (Sp1), 10q31-->q32.1 (Sp2), 3q24-->q31 (Sp3) and 6q33 (Sp4) and of the SP2 gene to human chromosome bands 17q21.3-->q22 by *in situ* hybridization, Cytogenet. Cell Genet. 81, 273-274.

53. Smidt M.P., van Schaick H.S.A., Lanctot C., Tremblay J., Cox J., van der Kleij A.A.M, Wolterink G., Drouin J., Burbach J.P.H., (1997). A homeodomain gene *Ptx3* has highly restricted brain expression in mesencephalic dopaminergic neurons, Proc Natl Acad Sci USA 94, 13305-13310.

54. Smidt M.P., Asbreuk C.H., Cox J.J., Chen H., Johnson R.L., Burbach J.P. (2000a). A second independent pathway for development of mesencephalic dopaminergic neurons

requires *Lmx1b*, Nat Neurosci 3,337-341.

55. Smidt M.P., Cox J.J., Schaick H.S., Coolen M., Schepers J., Kleij A.M., Burbach J.P. (2000b). Analysis of three *ptx2* splice variants on transcriptional activity and differential expression pattern in the brain, J Neurochem 75, 1818-1825.

56. Staines W.A., Morassutti D.J., Reuhl K.R., Ally A.I., McBurney M.W. (1994). Neurons derived from P19 embryonal carcinoma cells have varied morphologies and neurotransmitters, Neuroscience 58, 735-751.

57. Stephens R.M., Schneider T.D. (1992). Features of spliceosome evolution and function inferred from an analysis of the information at human splice sites, J. Mol. Biol. 228, 1124-1136.

58. Stork O., Ji F.Y., Kaneko K., Stork S., Yoshinobu Y., Moriya T., Shibata S., Obata K. (2000). Postnatal development of a GABA deficit and disturbance of neural functions in mice lacking GAD65, Brain Res. 865, 45-58.

59. Stuhmer T., Anderson S.A., Ekker M., Rubenstein J.L. (2002). Ectopic expression of the Dlx genes induces glutamic acid decarboxylase and Dlx expression, Development. 129, 245-252.

60. Szabo G., Katarova Z., Greenspan R. (1994). Distinct protein forms are produced from alternatively spliced bicistronic glutamic acid decarboxylase mRNAs during development, Mol. Cell. Biol. 14, 7535-7545.

61. Szeto D.P., Ryan A., O'Connell S., Rosenfeld M., (1996). P-OTX: A PIT-1-interacting homeodomain factor expressed during anterior pituitary gland development, Proc Natl Acad Sci USA 93, 7706-7710.

62. Szeto D.P., Rodriguez-Esteban C., Ryan A.K., O'Connell S.M., Liu F., Kioussi C., Gleiberman A.S., Izpisua-Belmonte J.C., Rosenfeld M.G. (1999). Role of the bicoid-related homeodomain factor Pitx1 in specifying hindlimb morphogenesis and pituitary development,

Genes Dev 13:484-494.

63. Urano Y., Watanabe K., Sakai M., Tamaoki T. (1986). The human albumin gene, characterization of the 5' and 3' flanking regions and the polymorphic gene transcripts, J. Biol. Chem. 261, 3244-3251.

64. Westmoreland J.J., McEwen J., Moore B.A., Jin Y., Condie B.G. (2001). Conserved function of Caenorhabditis elegans UNC-30 and mouse Pitx2 in controlling GABAergic neuron differentiation, J. Neurosci. 21, 6810-6819.

65. Yanagawa Y., Kobayashi T., Kamei T., Ishii K., Nishijima M., Takaku A., Kobayashi T., Tamura S. (1997). Structure and alternative promoters of the mouse glutamic acid decarboxylase 67 gene, Biochem. J. 326, 573-578.

Table 1

Sequences of primers used in RT-PCR and Southern hybridization experiments

Gene	Primer name	Sequence		Reference
VGAT	Primer-1	5'-TCGATGTGGCCATCTTCGTCATCGG-3'	(5823-5847)	GenBank AB080232
	Primer-2	5'-CGACCGGATACATTGCACAAGATGAGT-3'	(6511-6537)	GenBank AB080232
	Primer-3*	5'-CACTCGAGGGAAAGTTCGC-3'	(1573-1591)	(Sagne et al., 1997)
	Primer-4*	5'-CACTCGAGGGTCTCATCGA-3'	(5880-5898)	GenBank AB080232
	Primer-5	5'-CCAAGATCACGGCGTGG-3'	(2504-2520)	GenBank AB080232
	Primer-6	5'-AAGCGATGACCAGGATGT-3'	(5181-5198)	GenBank AB080232
	Primer-7*	5'-GTCCTGGTCCATCATAGC-3'	(5071-5088)	GenBank AB080232
GAD67	Primer-8	5'-TCCTGGTTGACTGTAGAGAC-3'	(615-634)	(Katarova et al., 1990)
	Primer-9	5'-AAGAGTAATCTGTTCCATGAG-3'	(782-802)	(Katarova et al., 1990)
	Primer-10*	5'-TTCACCAGCTAAACCAATGAT-3'	(701-721)	(Katarova et al., 1990)
HPRT	Primer-11	5'-GACCCGCAGTCCCAGCGTCG-3'	(93-112)	(Konecki et al., 1982)
	Primer-12	5'-CAAGACATTCTTTCCAGTTAAAGTTG-3'	(455-480)	(Konecki et al., 1982)

*Used for Southern hybridization.

A


```
E           B              B       E              N   E

        exon 1      intron 1          exon 2       I    exon 3
```

0.5 kb

B, *Bam*HI; E, *Eco*RI; N, *Not*I
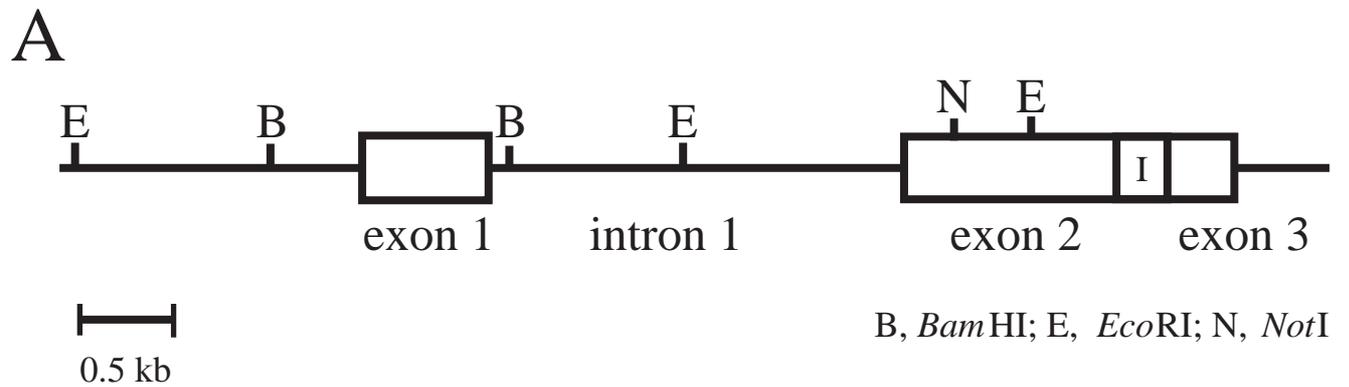
B

Exon		Intron			
No.	Size (bp)	5'-Donor site	No.	Size (bp)	3'-Acceptor site
1	599	ATTCAGgtgagtgc	1	2,185	caccacagGGCATG
2	1,150	TCGAGGgtctcatc	2	388	aagggaggGAAAGT
3	393				

Figure 1. Structure of the mouse VGAT gene.
A. Schematic representation of the mouse VGAT gene. The exons are represented by boxes. I is intron 2 in VGATa and is included in the transcript in VGATb.
B. Nucleotide sequences of the exon/intron boundaries of the mouse VGAT gene. Exon sequences are shown in capital letters and intron sequences in lowercase letters.

A

```
                        ┌────────────────────────Primer-1──────────────────────────►┐
     5816 │GTC TTC TTC GAT GTG GCC ATC TTC GTC ATC GGC GGC ATC TGC AGC GTG│   exon 2
         │ V   F   F   D   V   A   I   F   V   I   G   G   I   C   S   V │
     5864 │TCC GGC TTC GTG CAT TCA CTC GAG G│GT CTC ATC GAG GCC TAC CGA ACC
         │ S   G   F   V   H   S   L   E     G   L   I   E   A   Y   R   T
     5912  AAC GCA GAG GAC TAG    ggggtgggga ccctgccccc agctccatcc ccgcccaccc
          N   A   E   D   *
     5967  ccactccccg tcattcccgc ccccacccccc actcccagcc tcctgcgccg tcgcgctagg
     6027  gaggccgagc tttaaacacc tccggttcct agttgctgat tattcgggga ccgggtgggg
     6087  gagggagggg atagacatcc aaggtccact gcgtctgcgt ttctgtcgtc ctttctattc       I
     6147  cacatcgccc tgattggggg gagggagcgg agcgtattag tgaagggtat tttctgtcct
     6207  tcctggaaca ccccatcacc accaccacca aattttggct cctgtcaacg acggggggtgg
     6267  gaagggagg ┌GA AAG TTC GCA GGC TTG GAA ACT TGAccttgagggt ggggtggggc
                    │ G   K   F   A   G   L   E   T   *
     6322 │catttcacag ccattcagtg cttggaatct acagcgtcca gccatttcca gcaagagcgc
     6382 │tccccatgcc ctggacattt caaccttgcg gcctgaaagg ttgaccggga aatccatttc
     6442 │gggcgggcga cttcactctg gagaagccgc ggcaggggcc ccgtttgcct gccggtttttc   exon 3
                                  ◄──────────Primer-2───────
     6502 │aggaaccaaa ctcatcttgt gcaatgtatc cggtcgtgga actgtatact gtgcgtgtgg
     6562 │tgtgctcgtg gtgaataaga tgaaatgtat atcagaaaaa aatctatctc taatttagag
     6622 │tgcggtgcat aataattata tccgcaaata aagaagaaac aaaggcg
                            polyadenylation signal
```

B

```
                exon 2                                        exon 3
                               ┌──────VGATa probe──────┐
VGATa (521 a.a.)  TTC GTG CAT TCA CTC GAG GGA AAG TTC GCA GGC TTG GAA ACT TGA
                   F   V   H   S   L   E   G   K   F   A   G   L   E   T   *
                                       ↑                                 stop

                exon 2
                               ┌──────VGATb probe──────┐
VGATb (525 a.a.)  TTC GTG CAT TCA CTC GAG GGT CTC ATC GAG GCC TAC CGA ACC AAC GCA GAG GAC TAG
                   F   V   H   S   L   E   G   L   I   E   A   Y   R   T   N   A   E   D   *
                                       ↑                                             stop
```

Figure 2. Identification of VGATb as a new mouse VGAT mRNA.

A. Sequence and organization of the 3'-end of the mouse VGAT gene. Coding and noncoding sequences are shown in uppercase and lowercase letters, respectively. The exons are represented by boxes. I is intron 2 in VGATa and is retained in the VGATb transcript. The positions of Primer-1 and Primer-2 for RT-PCR (Fig. 4) are indicated by arrows. The stop codons are indicated by asterisks.

B. Different C-terminal amino acid sequences of VGATa and VGATb. The start sites of the different nucleotide sequences between VGATa and VGATb by splicing are indicated by vertical arrows. The positions of the VGATa and VGATb probes for Southern blotting (Fig. 4) are indicated by horizontal lines.

A

VGAT gene

| 1 | | 2 | I | 3 |

Primer-1 →   ← Primer-2

VGATa

| 1 | 2 | 3 | **328 bp**

Primer-1 →   ← Primer-2
VGATa probe

VGATb

| 1 | 2 | I | 3 | **715 bp**

Primer-1 →   ← Primer-2
VGATb probe

B

bp   1   2   3

1230 —

653 —   ←715

394 —

298 —

C

1   2   3

←328

VGATa probe

D

1   2   3

←715

VGATb probe
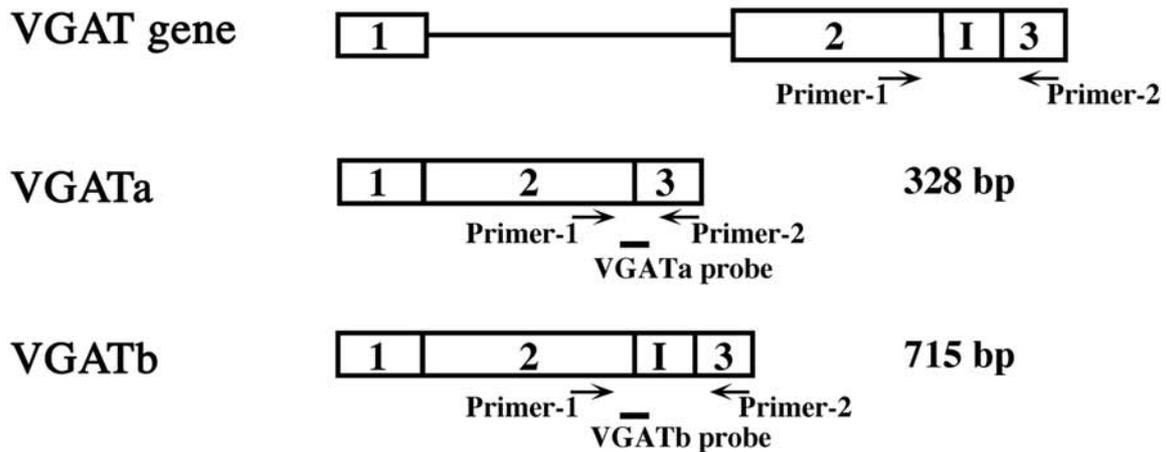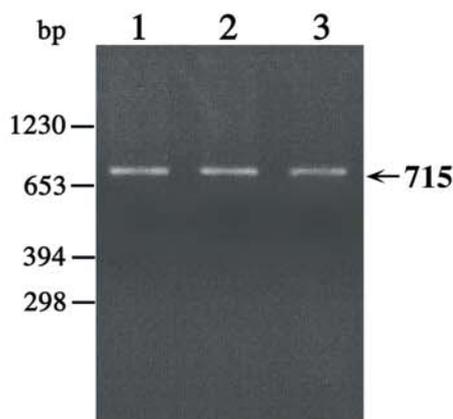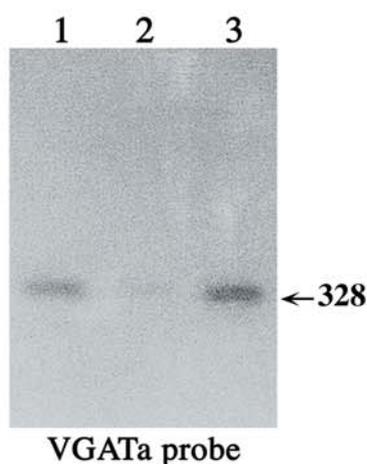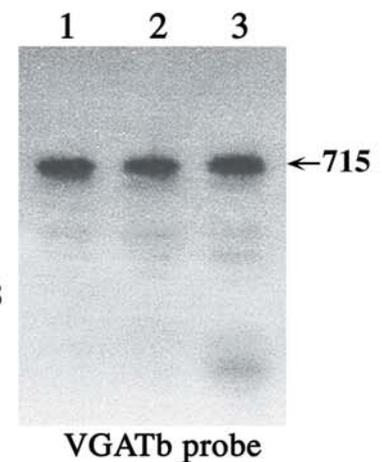
Figure 3. Detection of mouse VGATa/b mRNAs in the mouse CNS.

A. Schematic representation of the mouse VGAT gene and of the alternative splicing processes for VGAT mRNAs. The arrows indicate the locations of the primer sets (Primer-1 and Primer-2) used to amplify the two different alternatively spliced VGAT isoforms VGATa and VGATb. The sizes of the expected RT-PCR products are shown on the right. The digoxigenin-labeled VGATa and VGATb probes were used to detect RT-PCR products specific to the VGATa and VGATb isoforms, respectively.

B. Dominant expression of VGATb in the mouse CNS. RNA from mouse cerebrum (lane 1), cerebellum and brainstem (lane 2), and spinal cord (lane 3) was reverse-transcribed and then amplified using a forward primer (Primer-1) specific for exon 2 and a reverse primer (Primer-2) in exon 3. The RT-PCR products were analyzed by electrophoresis on a 1.5% agarose gel. DNA size standards in base pairs (bp) are indicated on the left side.

C, D. Southern blot analysis of RT-PCR products shown in (B) to confirm VGATa (C) and VGATb (D) expression. The numbers on the right side indicate the sizes of the PCR products in base pairs.

```
-1954 gaattcctcc --------- 1,320 bp ---------cttc aaaagtaatc caagacagtc acttctgtca tcaggatgg
                                                       Pitx
 -580 cttgcagaac aagtgataga tggcagagac acaaacagac gaccgatcgg ccggcctagc tctagagact ctcacctgt

 -500 tgtcctgttg gttatccgga cggttagcca gaggatccgg ggcccccgca cagctccggg actctggaag atagttccg
                                                  Egr-1
 -420 gggtgggggac cttcgagaac cagcccacac tgaactcctc cctccttgtg gcagcagcaa ggtgggacgg gccaggacg

 -340 ctgcttagca cctcctccag aaatgcagca cttggggggc ccccaccctt tcgcgcgctc cttcccaccg acctcccag
                                                  Egr-1
 -260 ggtgcacctc tccagcctcg gtcgcgcttc cgaaaccttt ggtgcccccct tttcctggtc ccgacccccc acctcacgc

 -180 ccctggtctg gacagcatct ccccctcgcc gccctccgcc cacgcaccgc ctgactccga ggggtgcgag cgcattggg
                                   Sp1           Egr-1       Sp1                      CCAAT box
 -100 tgcgcccgcg tgggggcgcc gcgccagcct cgcgtagctg ttctgacgct gccgtcgccg ccgccct ccg cagcccagc
                Egr-1                                                         Sp1
  -20 ggcacccgca ccagctctgc agtgcactcg tcgcctctcg ggccggtccc accaagagcc agactgtcgt gaccggggc
                          *

  +61 agcctcgaac gtcaagcgcg agggtcatga gccagagcgc cctggggcgc cgcgcggaga cccagcggag atagcagtc

 +141 tcgctgcctt gacgcgcgcc cgccgcgtcc ccagacccct ctgtcctttt ctcccgcccc gccgccgcc ATG GCC AC
                                                                               M   A   T
```

Figure 4. Nucleotide sequence of the 5'-flanking region of the mouse VGAT gene.
Nucleotides are numbered relative to the major transcription start site (+1), which is indicated by an asterisk. Potential consensus sequences for regulatory elements CCAAT box and transcription factor (Egr-1, Sp1 and Pitx) binding sites are underlined. The coding sequences are written in uppercase letters and deduced amino acid sequence is shown below.
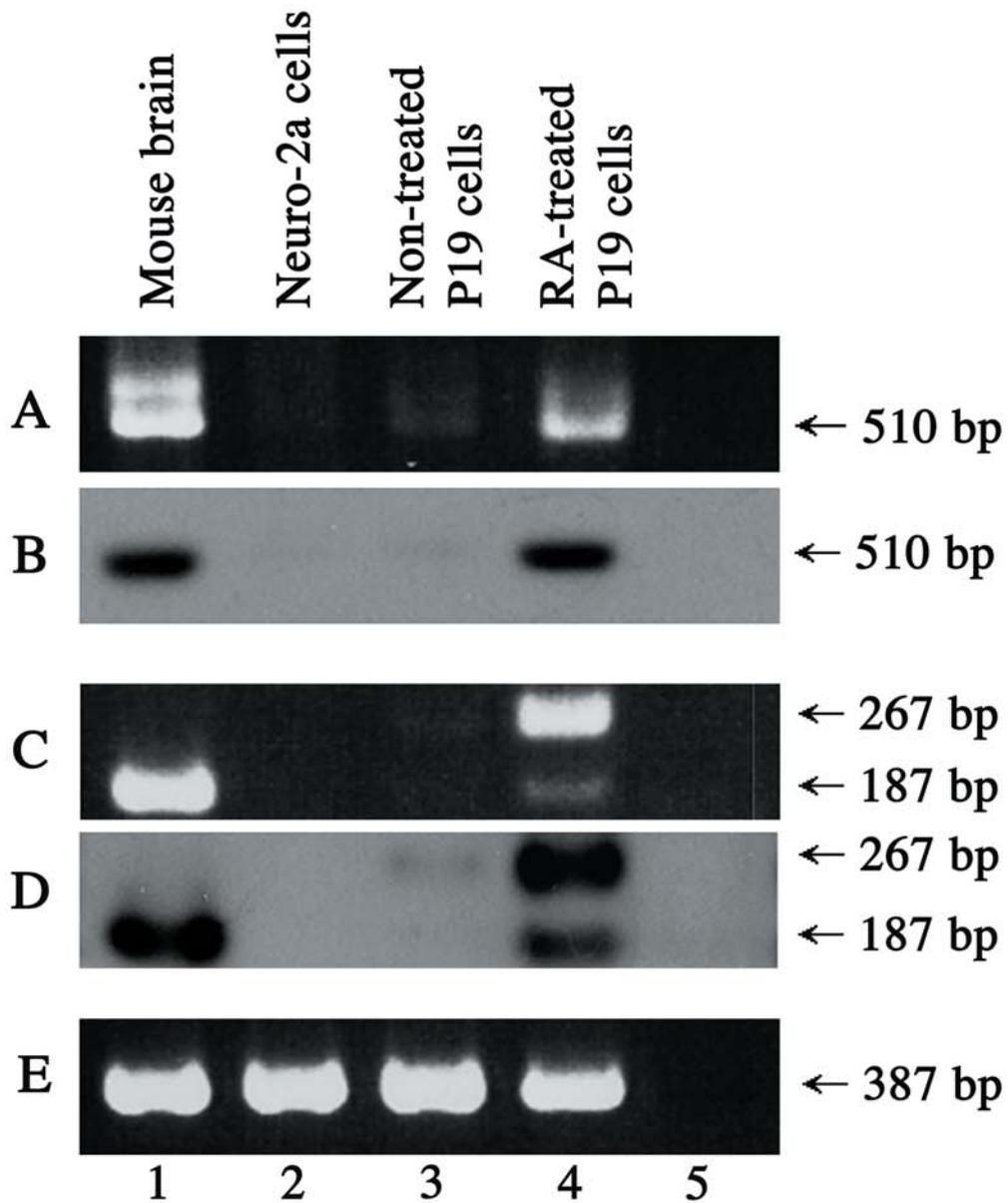
Figure 5. RT-PCR analyses of VGAT (A, B) and GAD67 (C, D) gene expression.
RT-PCR products (A, C and E) and their Southern blotting for VGAT (B) and GAD67 (D). The transcript of the HPRT gene (E) was amplified as a positive control. Lane 1, adult mouse brain; lane 2, Neuro-2a neuroblastoma cells; lane 3, non-treated P19 cells; lane 4, RA-treated P19 cells; lane 5, mock reverse-transcribed RNA prepared from RA-treated P19 cells was also amplified. The numbers at the right indicate the sizes of the PCR products in base pairs.
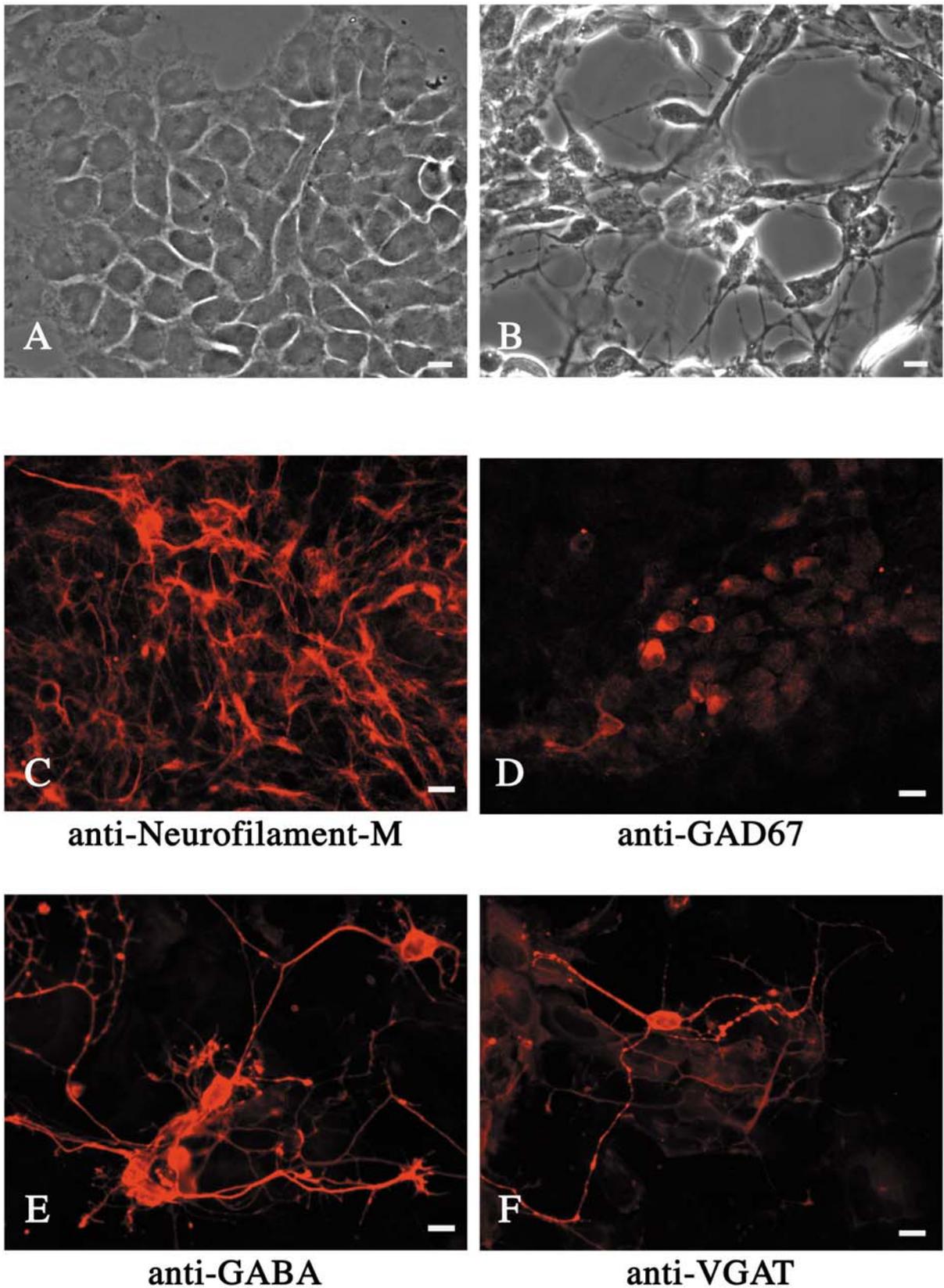
Figure 6. Immunohistochemistry of RA-treated P19 cells.

A and B. Phase-contrast micrographs of non-treated P19 cells (A), and P19 cells treated with 5 μM RA for 8 days (B).

C-F: RA-treated P19 cells were immunostained with anti-neurofilament-M (C), anti-GAD67 (D), anti-GABA (E), or anti-VGAT (F) antibodies. The bars indicate 10 μm.
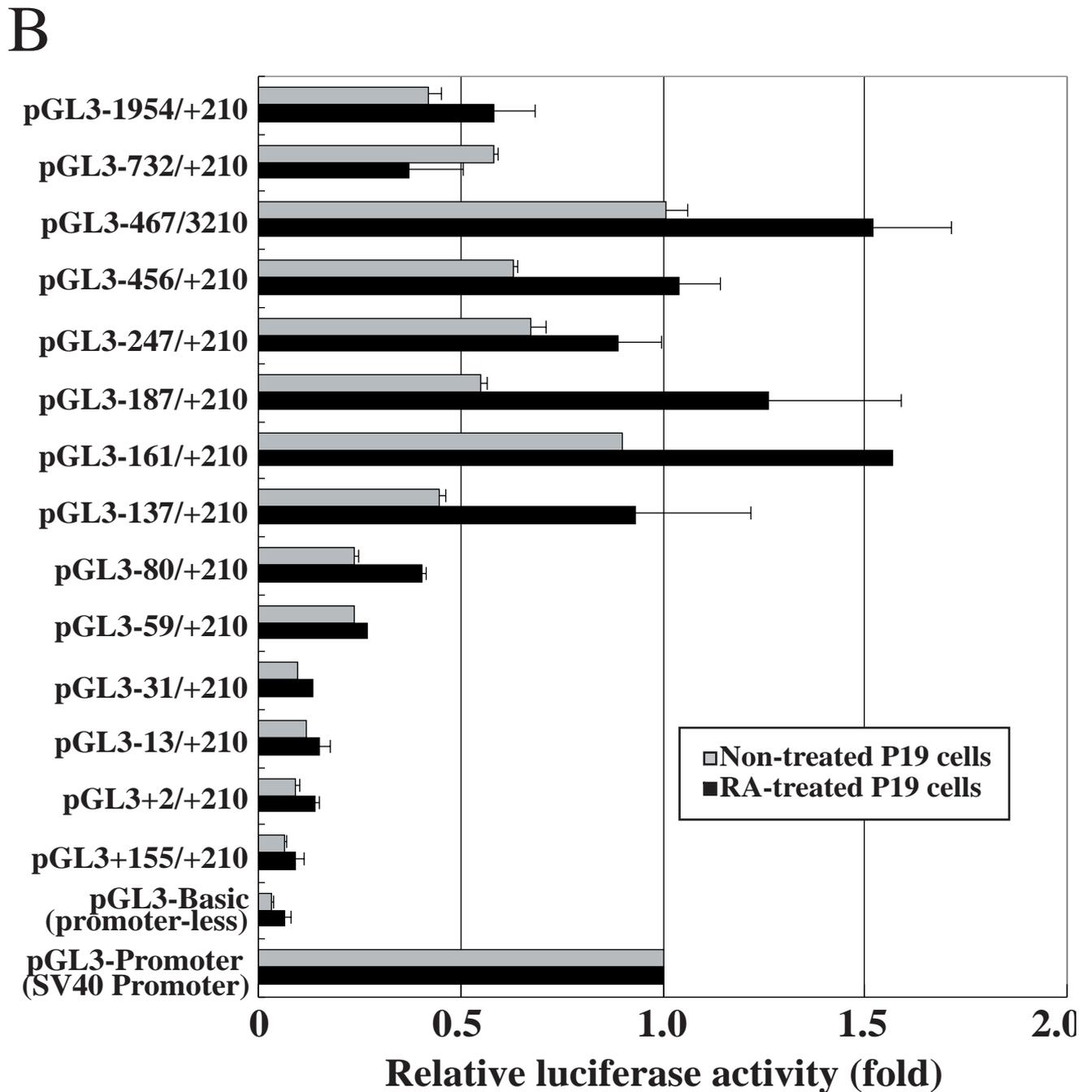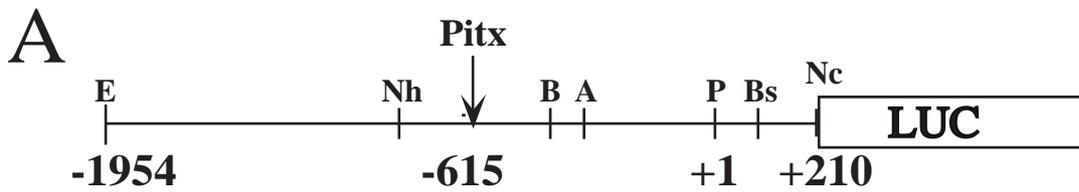
Figure 7. Expression of luciferase activities in RA-treated and non-treated P19 cells.
A. Structure of the mouse VGAT gene. The numbers are counted from the transcription start site (+1). A, *Apa*I; B, *Bam*HI; Bs, *Bss*HI; E, *Eco*RI; Nc, *Nco*I; Nh, *Nhe*I; P, *Pst*I; Pitx, Pitx binding site.
B. Expression of luciferase activities in RA-treated P19 cells and non-treated P19 cells.
Deletion constructs containing different lengths of the VGAT 5'-flanking region were transfected into non-treated P19 cells (gray) or RA-treated P19 cells (black). The position numbers are counted from the transcription start site. The plasmids pGL3-Basic and pGL3-SV40 were used as a negative and a positive control, respectively. The luciferase activity (fold) was calculated relative to the pGL3-Promoter activity. SV40, simian virus 40. Values are given as the mean ± SE of three independent transfection expriments.

A

Oligo I: 5'-$\overset{-49}{\text{ccgtcgc}}\underline{\text{cgccgccct}}\overset{-27}{\text{ccgcagc}}$-3'

Sp1

B

Competitor: Sp1   Oligo I   NF-Y

None ×20 ×200 ×400 ×20 ×200 ×2,000 ×2,000

1 2 3 4 5 6 7 8
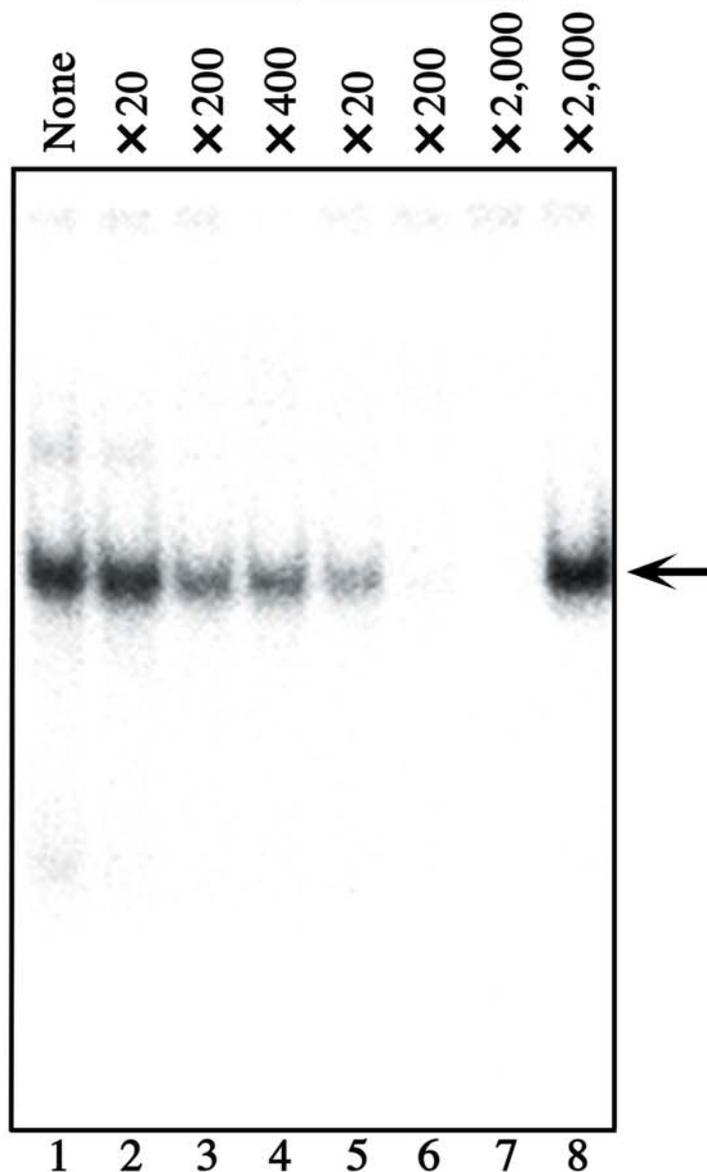
Figure 8. Gel shift assay of P19 cell crude nuclear extracts with the mouse VGAT promoter including Sp1 site.
A. Sequences of the oligonucleotides used in gel shift assays. Oligo -49 to -27 of the mouse VGAT promoter was named Oligo I.
B. Labeled Oligo I was incubated with non-treated P19 crude nuclear extracts in the absence or presence of unlabeled competing oligonucleotides. Lane 1, without competitor oligo; lanes 2-4, with 20-, 200-, or 400-fold molar excess of unlabeled consensus Sp1 competing oligonucleotide; lanes 5-7, with 20-, 200-, or 2,000-fold molar excess of unlabeled Oligo I competing oligonucleotide; lane 8, with 2,000-fold molar excess of unlabeled NF-Y competing oligonucleotide. An arrow indicates the Oligo I-DNA complex.
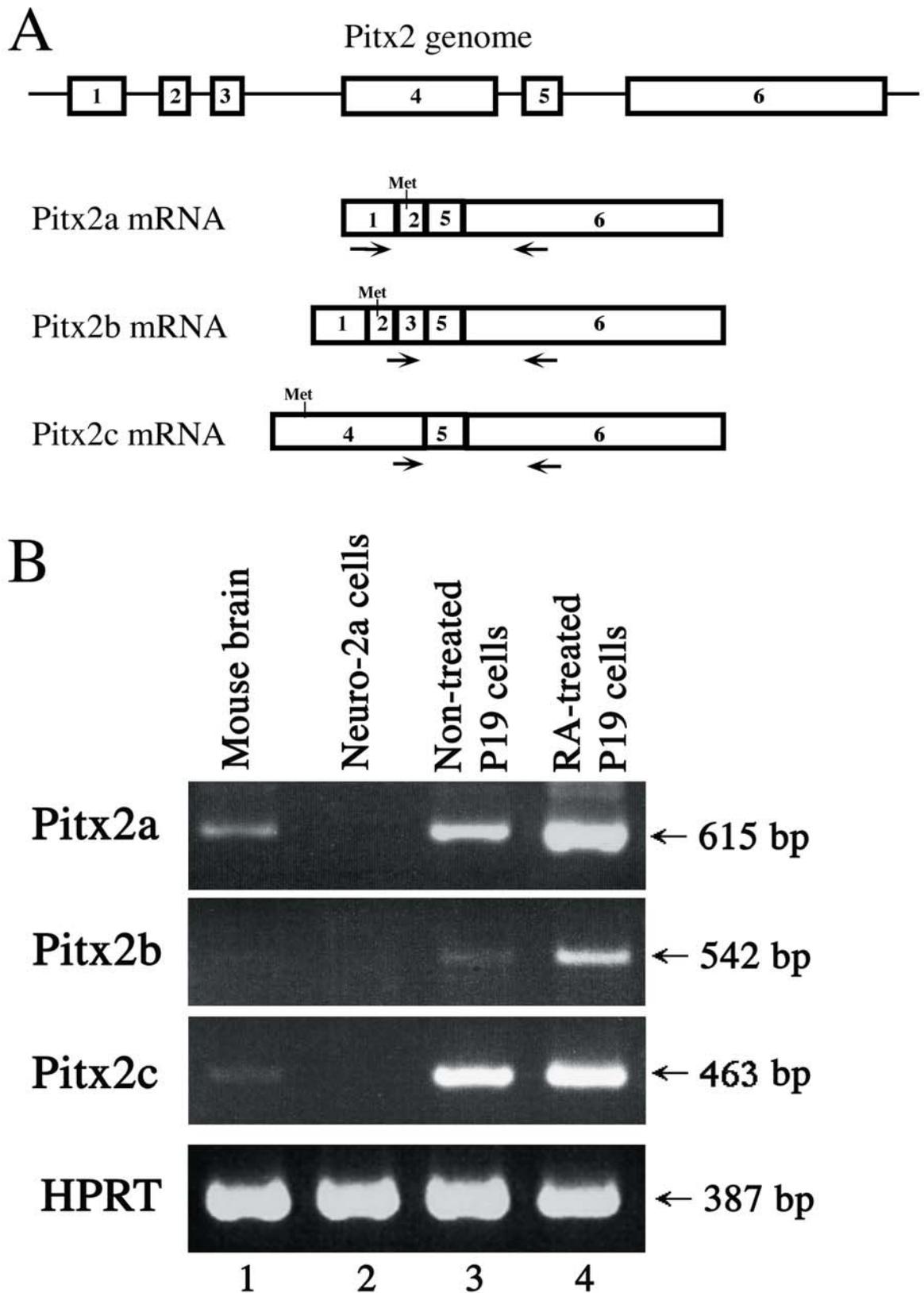
Figure 9. Expression of mouse Pitx2a/b/c mRNAs.
A. Schematic representation of the mouse Pitx2 gene and of the alternative splicing
process for Pitx2 mRNAs (Kitamura et al., 1997). The exons are represented by boxes.
The location of primers to amplify the three different alternatively spliced Pitx isoforms,
Pitx2a, Pitx2b and Pitx2c are shown by arrows.
B. The expression of Pitx2a, Pitx2b and Pitx2c in adult mouse brain (lane 1), Neuro-2a
neuroblastoma cells (lane 2), non-treated P19 cells (lane 3), and RA-treated P19 cells (lane
4) was analyzed by RT-PCR. The expression of HPRT was used as a positive control. The
numbers indicate the sizes of the PCR products in base pairs.
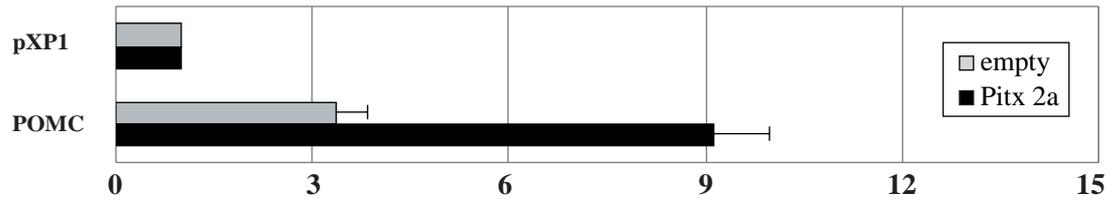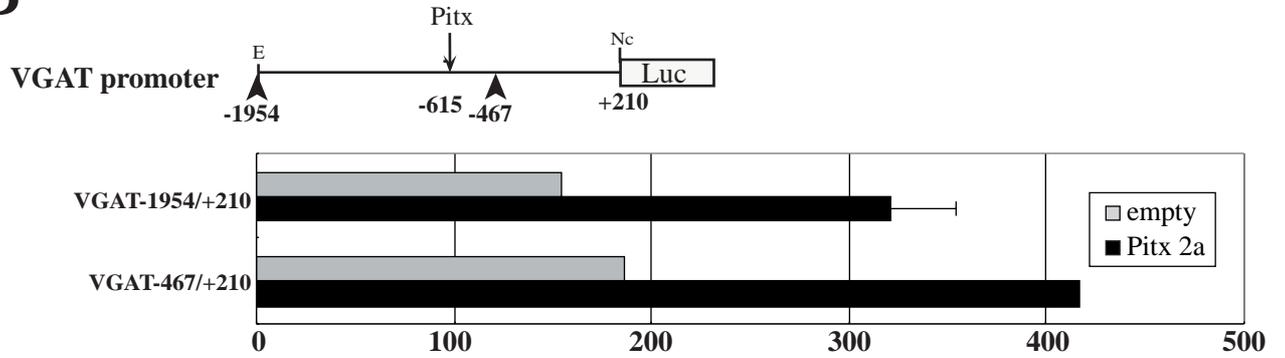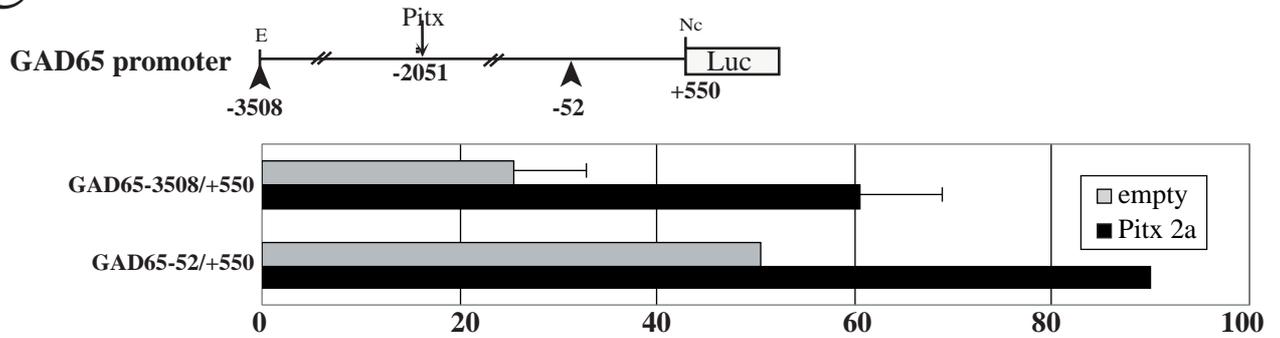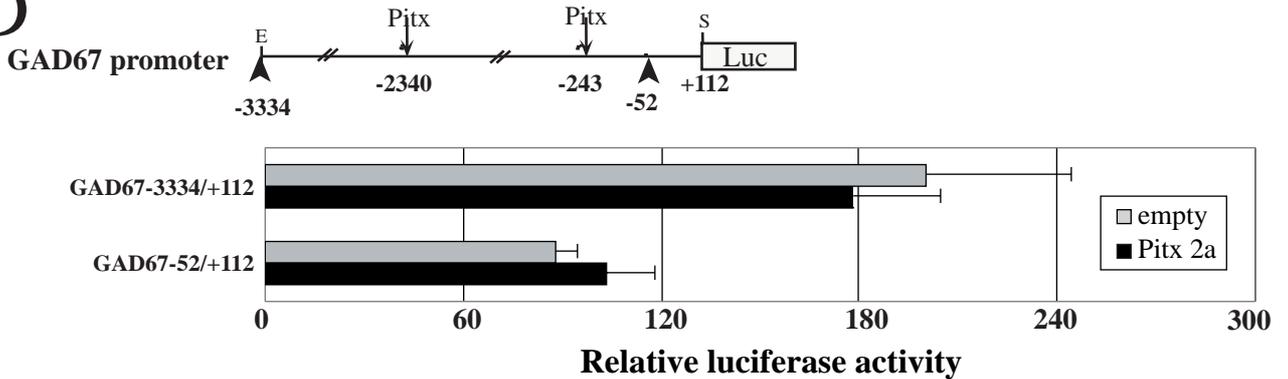
Figure 10. Effect of Pitx2a on POMC (A), VGAT (B), GAD65 (C) and GAD67 (D) promoters in RA-treated P19 cells.

A. Shows a negative and a positive control obtained by the plasmids pXP1 and POMC, respectively. Schematic of the VGAT, GAD65 and GAD67 promoter constructs is presented at the top of B-D, respectively. *Arrowheads* indicate the positions of the deletion constructs.

A-D. The relative luciferase activity of pXP1 and POMC (A), VGAT (B), GAD65 (C) and GAD67 promoter luciferase constructs (D) obtained with the promoter-less pXP1 vector measured as 1 in the presense of coexpressed RSV-Pitx2a expression plasmid (Pitx2a: black) or the RSV plasmid without Pitx2a (empty: gray). Values are given as the mean ± SE of three independent transfection experiments.