Structure and alternative promoters of the mouse glutamic acid decarboxylase 67 gene

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γ-Aminobutyric acid is synthesized by glutamic acid decarboxylase (GAD), which has two forms, GAD65 and GAD67. Genomic clones coding mouse GAD67 (mGAD67) have been isolated. The restriction map of the overlapping clones covers a region of more than 45 kb of genomic DNA. The mGAD67 gene contains 16 translated exons in addition to an exon which is preferentially expressed in foetal brain. The rapid amplification of 5′-cDNA ends showed that mGAD67 gene transcripts have two different 5′-untranslated regions. Analysis of the genomic clones encompassing the 5′-exons revealed that the two transcripts arose from a single gene by alternative splicing using two different donor sites and a common acceptor. The exons were found 1.5 and 0.6 kb upstream of exon 1. The corresponding promoter regions of these exons have a number of putative regulatory elements, including Sp1- and Krox-24-binding sites. Analysis of mGAD67 transcripts demonstrated that each of the 5′-untranslated exons was expressed in mouse brain. In contrast, exon 0A, but not exon 0B, was expressed in mouse testis and pancreas. These results suggest that these transcripts may be regulated under the control of independent promoters.

INTRODUCTION

γ-Aminobutyric acid (GABA) is considered to be a principal inhibitory neurotransmitter and GABAergic neurons comprise more than 30 % of all neurons in the adult mammalian central nervous system.

Glutamic acid decarboxylase (EC 4.1.1.15; GAD) is the rate-limiting enzyme that catalyses the production of GABA from glutamic acid [1]. The two enzymes, GAD65 and GAD67, encoded by separate genes, have been found in mammalian brain [2,3]. They can be distinguished by their molecular masses as well as by their cofactor interactions and subcellular distributions [4,5]. GAD65 is largely membrane-bound and is relatively enriched in vesicular membranes, whereas GAD67 is soluble and is distributed in the cytoplasm. GAD67 cDNAs have been isolated from several species, including human [6], rat [7], mouse [8] and cat [9]. The amino acid sequence of GAD67 is highly conserved among these species. Mouse GAD67 (mGAD67) cDNA encodes a protein of 515 amino acids that is preceded by at least approx. 200 bp of 5′-untranslated region (5′-UTR) sequence [8]. Analysis of the partial genomic structure revealed the presence of an intron within the 5′-UTR [8]. The mGAD67 gene is located on chromosome 2 [10].

GAD is expressed primarily in GABAergic neurons of the adult mammalian central nervous system, but it is also present in pancreatic β-cells and testis [11,12]. During embryonic development, GAD67 is detectable at an early stage [13]. GAD67 expression is modulated by pharmacological treatment with drugs. Injection of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) into monkeys results in increases in GAD67 mRNA levels [14]. Agonists of dopamine D_2 receptors reduce GAD67 mRNA levels, whereas antagonists of D_2 receptors increase its levels in rat striatum [15]. Cyclic AMP decreases GAD67 expression in C6 cells [16]. Kainic acid-induced seizure results in a marked increase in GAD67 expression in rat hippocampal granule cells [17].

We analysed mGAD67 cDNAs and identified a cDNA clone that differed from the previously determined mGAD67 sequence at the 5′-end of the clone. The two sequences diverged at the point where exon 0 is spliced on to exon 1. We have characterized exon/intron organization, alternative splicing products and 5′-flanking regions of the mGAD67 gene to investigate the regulatory mechanisms controlling transcription of this gene.

MATERIALS AND METHODS

Isolation and characterization of genomic clones

A mouse 129SV genomic library in the λFIXII vector (Stratagene, La Jolla, CA, U.S.A.) was screened by plaque hybridization using a 1.8 kb cloned fragment of mGAD67 cDNA as the probe. Nine overlapping plaques covering the region from exon 3 to the 3′-region were isolated. A 0.2 kb cloned fragment (corresponding to exon 0 and part of intron 0) was used to screen and identify the 5′-region. A recombinant phage clone λ105 extending from the 5′-flanking region to exon 1 was isolated. Another screening was performed using a *Sac*I fragment of the λ105 clone (corresponding to intron 0 and exon 1) and a recombinant phage, λ141, extending from intron 0 to exon 4, was isolated. Positive plaques were purified, and DNA was isolated by the bacteriophage lysate method [18]. Six overlapping phages, λ2, λ5, λ23, λ27, λ105 and λ141, covering the whole gene, were subcloned in pBluescript SK(−) (Stratagene, La Jolla, CA, U.S.A.) using

Abbreviations used: GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase; mGAD67, mouse GAD67; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; poly(A)$^+$, polyadenylated; RACE, rapid amplification of 5′-cDNA ends; RT-PCR, reverse transcriptase PCR; 5′-UTR, 5′-untranslated region.

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The nucleotide sequence data reported will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases under the accession number D88826.

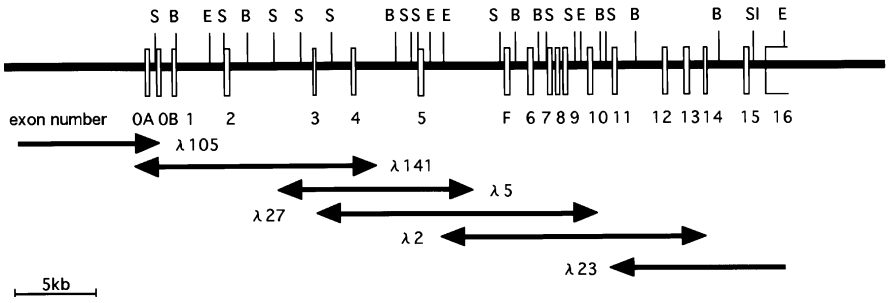

Figure 1 Schematic representation of the mouse GAD67 gene

Restriction enzymes are abbreviated as follows: B, *Bam*HI; E, *Eco*RI; Sc, *Sac*I; SI, *Sal*I. Exons are represented by boxes, whereas introns and flanking sequences are represented by solid lines. The exon in which the translated region begins is designated exon 1 and the exons before exon 1 are called exon 0A or exon 0B. Overlapping inserts from the λ genomic clones are shown at the bottom.

*Not*I or *Sal*I of the λFIXII polylinker and an internal *Not*I or *Sal*I site to create plasmids for further analysis.

Nucleotide sequence analysis

cDNA clones, genomic clones and PCR products were digested with restriction enzymes, subcloned into pBluescript SK(−) and sequenced by the dideoxy method [19].

Preparation of RNA

Total cellular RNA was prepared from mouse brain, testis or pancreas by an established method [20]. Polyadenylated [poly(A)$^+$] RNA was prepared using Oligotex-dT30 (TaKaRa, Otsu, Japan).

Rapid amplification of 5′-cDNA ends (5′-RACE)

cDNA was synthesized from 1 μg of the mouse brain total RNA using Superscript reverse transcriptase (Life Technologies, Gaithersburg, MD, U.S.A.) and the primer G#1, 5′-ACAGATCTTCAGGCCCAGTT-3′, an antisense oligonucleotide corresponding to nucleotides +237 to +256 of mGAD67 cDNA [8]. After addition of a poly(C) tail with deoxynucleotidyl transferase (Life Technologies, Gaithersburg, MD, U.S.A.), the cDNA was amplified using 5′-CUACUACUACUAGGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′ as anchor primer (Life Technologies) and the primer G#2, 5′-GCAGGTTGGTAGTATTAGGA-3′, which corresponds to nucleotides +166 to +185 of mGAD67 cDNA [8]. PCR was performed for 33 cycles of 1 min at 94 °C, 1 min at 60 °C and 2 min at 72 °C. PCR products were separated on a 2% agarose gel, identified by Southern blotting and subcloned into the pBluescript SK(−) vector.

Reverse transcriptase PCR (RT-PCR)

cDNA was synthesized using reverse transcriptase in a reaction mixture containing 1 μg of poly(A)$^+$ RNA from mouse brain, testis and pancreas. The equivalent of one fiftieth of the cDNA was then amplified using the G#1 primer from the mGAD67 common sequence and the other G#3 primer, which is specific to the exon 0A sequence (5′-AAGCAAGGAAGCAGCCCTG-3′ corresponding to nucleotides +39 to +57; see Figure 4A) or G#4, which is an exon 0B-specific primer (5′-ACTCTTCTATCCGAGGTCTC-3′ corresponding to nucleotides +18 to +37; see Figure 4B). DNA was amplified using ExTaq polymerase (TakaRa, Otsu, Japan). The reactions were denatured at 94 °C for 1 min before undergoing 33 cycles of 30 s at 94 °C, 1 min at 58 °C and 2 min at 72 °C. PCR products were run on a 2% agarose gel, transferred to nylon membranes and detected by hybridization to the G#2 oligonucleotide.

RESULTS AND DISCUSSION

Cloning and exon/intron junctions

Recombinant λ phages carrying the mGAD67 gene were isolated from a mouse genomic library. An analysis of the regions in six recombinant overlapping λ clones revealed that the coding region of the gene is spread over 45 kb. A complete restriction map of the clones was prepared for the restriction endonucleases *Bam*HI, *Eco*RI, *Sac*I and *Sal*I (Figure 1). The gene is divided into 19 exons (Figure 1), and all nucleotide sequences flanking the exons (Table 1) match the eukaryotic 5′-donor and 3′-acceptor consensus splice junction sequence GT-AG [21,22]. Compared with the structure of the human GAD67 gene, all of the exon/intron junctions are located at the same sites, except for exon 0B and exon F, which has not been described in humans [23]. The mGAD67 gene is a unique gene [10] which gives rise to mRNA variants generated by alternative splicing [24]. mGAD67 variants contained 80 or 86 bp and their expression is developmentally regulated; they are preferentially expressed in foetal brain [24]. In the mGAD67 gene, these foetal mRNA variants are encoded by exons 5 and 6, and were named exon F (Figure 1 and Table 2).

5′-Untranslated regions

The 5′-RACE method was used to analyse the 5′-ends of mGAD67 transcripts expressed in mouse brain. Several of the clones obtained using the 5′-RACE method had sequences identical to the one previously found in mGAD67 cDNA [8]. The remainder contained a novel form of the mGAD67 transcript, which diverged at the same point as other cDNAs, indicating that two different exons are alternatively spliced at this site. We nominated the 5′-sequence found in the published cDNA clone as exon 0A. The novel sequence obtained from the 5′-RACE clones was named exon 0B. The analysis of cloned λ phage genomic DNA revealed that exon 0A and exon 0B are located 1.5 kb and 0.6 kb upstream of exon 1, respectively (Figure 2).

There are a number of genes which have both alternative promoters and splicing in the 5′-UTR, including the genes for betaine γ-amino-n-butyric acid transporter [25], aromatase cytochrome *P*-450 [26] and acetylcholinesterase [27]. It is probable

Table 1 Nucleotide sequence of the exon/intron limits of the mouse GAD67 gene

Exon sequences are shown in capital letters and intron sequences in lower-case letters. The conserved splice donor (GT) and splice acceptor (AG) sites present in the intron borders are shown in bold type. The estimated size of each intron is indicated in kb.

Exon		Intron.			
No.	Size (bp)	5′-Donor site	No.	Approx. size (kb)	3′-Acceptor site
0A	> 129	ACCGAG **gt** gggtaa	0A	1.5	gtttgc **ag** CCTGTT
0B	> 258	TGCAAG **gt** gactga	0B	0.6	gtttgc **ag** CCTGTT
1	143	CTACAA **gt** ggtttc	1	3.3	gctcct **ag** CGTATG
2	63	TCTGTG **gt** aagtaa	2	5.6	cacttt **ag** GCTTCT
3	159	CTCAAG **gt** ggtttc	3	1.6	ctgctt **ag** ATCTGC
4	243	GCACAG **gt** aagagt	4	4.8	ctctct **ag** GTCACC
5	91	CAATAT **gt** aagtct	5	5.9	ttattc **ag** GCCATC
F	86	GTGATG **gt** aactga	F	2.4	cactct **ag** GTTCAC
6	113	CTCCTG **gt** gagttg	6	0.9	gttgat **ag** GGGGAG
7	116	GAACAC **gt** gagttg	7	0.1	tgttgc **ag** AGTCAC
8	80	TGAAAG **gt** aggcag	8	0.2	aattat **ag** GGGGAA
9	55	CAAAAG **gt** aggtat	9	2.3	ccttgt **ag** GGCTAT
10	117	GTGGAT **gt** aagtgt	10	1.2	ctttgc **ag** GGCCAA
11	65	AGAAAG **gt** atcgaa	11	3.4	tcttcc **ag** GGCCAA
12	79	GAAAAG **gt** ttgtat	12	1.1	tatgac **ag** GGCCAA
13	150	GCAAAG **gt** atggag	13	2.1	tgaaac **ag** GGCACC
14	108	GGTGAG **gt** aggttg	14	2.6	ttctgc **ag** CCTGAG
15	90	CACAGG **gt** aaggat	15	0.9	atctca **ag** GTGGCT
16	> 1052				

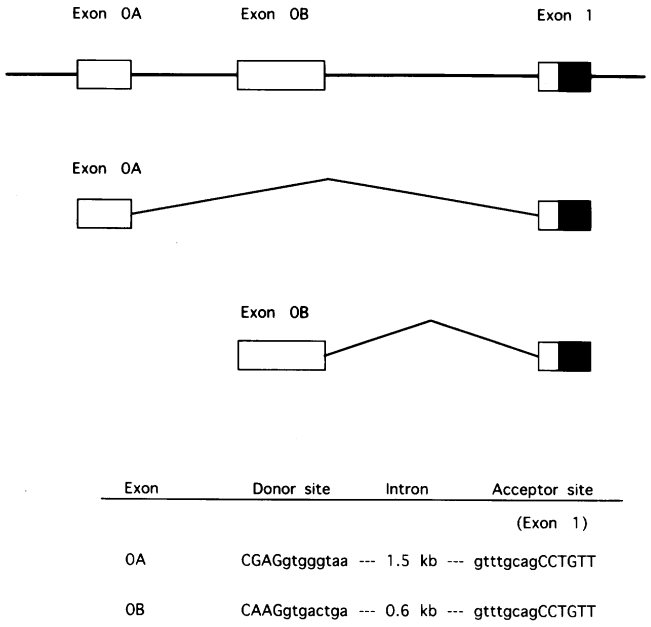

Figure 2 Exon/intron structure and splice sites in the 5′-UTRs of the mouse GAD67 gene

Open boxes represent untranslated sequence, and filled boxes represent the coding region of the gene.

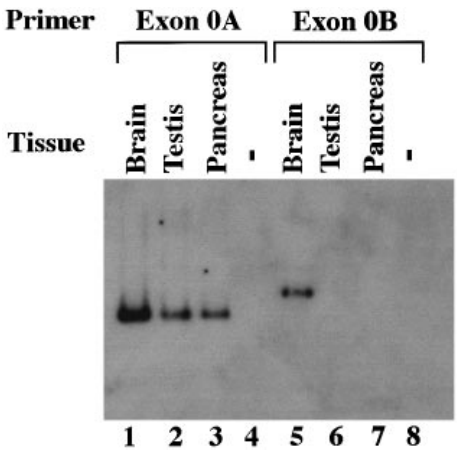

Figure 3 Expression of the mouse GAD67 5′-exons in mouse brain and testis

RNA from mouse brain (lanes 1 and 5), testis (lanes 2 and 6) and pancreas (lanes 3 and 7) was reverse-transcribed and then amplified using a 5′-exon-specific primer and a primer for exon 2. The RT-PCR products were analysed by electrophoresis on a 2% agarose gel. Lanes 1–4 contained a primer specific for exon 0A and lanes 5–8 contained the exon 0B-specific primer. PCR products were detected by hybridization to an internal oligonucleotide (G#2). Lanes 4 and 8 are negative controls containing no template.

that these genes encode the same polypeptide product and differ only in their 5′-UTR sequences. Possible functions of the 5′-UTR include modulating mRNA degradation [28] and regulating translation [29,30]. Therefore, the 5′-UTR of mGAD67 transcripts may be involved in regulating its translatability and/or stability.

Tissue-specific expression of 5′-exons

Expression of the alternative 5′-exons was analysed by RT-PCR in brain, testis and pancreas (Figure 3). The mRNAs from brain, testis or pancreas were reverse-transcribed using an oligo(dT) primer. The cDNA was then amplified using the mGAD67 exon 2 primer and a primer specific for exon 0A or exon 0B. PCR products were detected by hybridization to an internal oligonucleotide. mGAD67 transcripts containing exon 0A were de-

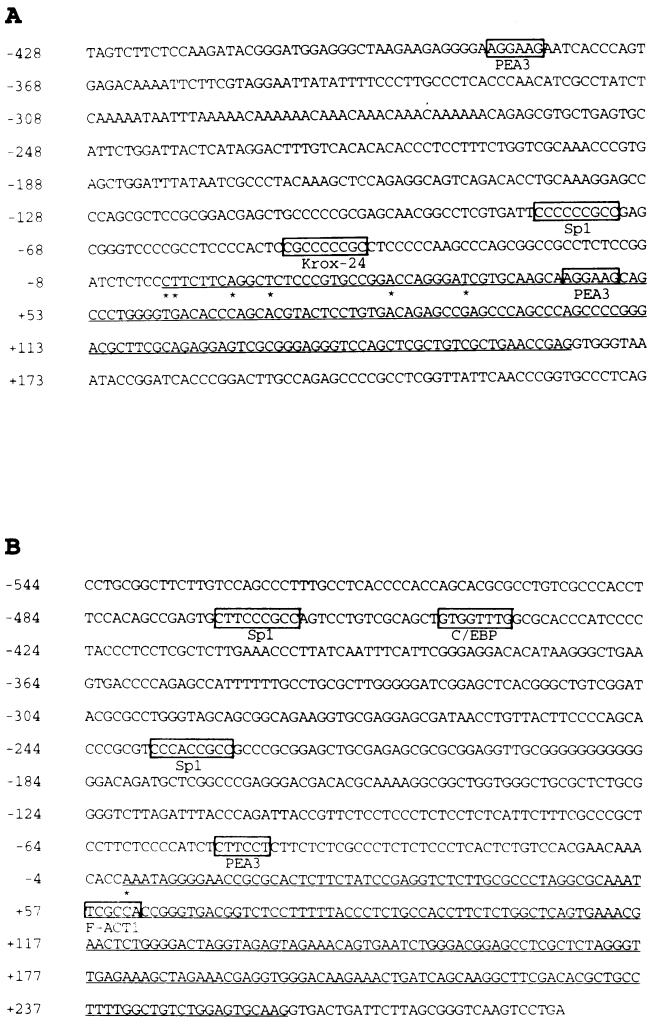

Figure 4 5′-Exon and -flanking sequences of the mouse GAD67 gene

The 5′-flanking sequences of exons 0A (**A**) and 0B (**B**) are shown along with the exon sequences. The exons are underlined, and numbering begins at the major transcription start site for each exon. The asterisks indicate the position of the 5′-ends of 5′-RACE clones. Abbreviations: PEA3, PEA3-binding site; Sp1, Sp1-binding site; Krox-24, Krox-24-binding site; C/EBP, binding sites for CCAAT-box-enhancer-binding protein; F-ACT1, F-ACT1-binding site.

tected in brain, testis and pancreas. Exon 0B was detected only in brain, but transcripts containing exon 0B were not detected either in testis or in pancreas RNA. These results demonstrate that exon 0B was expressed in brain, and not in testis or pancreas, indicating that promoter B is activated in a tissue-specific manner.

The majority of the 5′-RACE clones from brain contained the exon 0A sequence at their 5′-ends. These results suggest that the transcript containing exon 0A is the most abundant mGAD67 transcript in the tissues analysed. However, it remains to be determined which of these two 5′-exons represents the major transcript in other tissues, including spinal cord and foetal brain.

Analysis of 5′-flanking sequences

To aid the identification and characterization of the different promoters, we determined the nucleotide sequence of the 5′-flanking regions of each of the 5′ exons (Figures 4 and 5). We found that promoter A has a high G+C content (more than 70%) in the 180 bp preceding the transcription start site. Promoter A contains a non-canonical TATA box (TATAA) at position −170. However, it is likely that the TATA box may not be functional because of the existence of too many nucleotides between the TATA box and the transcription start site. Promoter A also contains a PEA3-binding site, an SP1-binding site and a Krox-24-binding site. Promoter B lacks a TATA box. However, it contains two Sp1-binding sites, a C/EBP-binding site and a PEA3-binding site. No CCAAT box was found in either promoter.

A sequence comparison between the promoter A region of mouse and human GAD67 [31] showed high similarity, suggesting the presence of similar regulatory elements for the expression of this gene (Figure 5). Interestingly, the position of the Krox-24-binding site in the mouse and human GAD67 gene promoter is conserved (Figure 5). However, the former is identical to a classic Krox-24-binding site, whereas the latter has one mismatch from the canonical site of Krox-24 [32]. The Krox family factors, which contain zinc-finger motifs, are encoded by immediate-early genes which are induced by diverse signals, such as growth factors and mitogens [33]. Krox-24 transcripts are very abundant

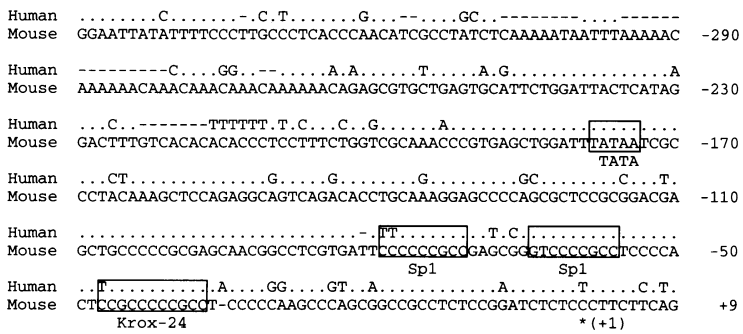

Figure 5 Comparison of nucleotide sequences of the GAD67 5′-flanking region between human and mouse genes

Numbers to the left of the sequences indicate the nucleotide positions in relation to the putative transcription start site. A non-canonical TATA box and consensus sequences for the putative regulatory elements (Krox-24 and Sp1) are boxed. Dots indicate identical nucleotides, whereas dashes indicate nucleotide deletions.

in the brain [34]. The aldolase C gene is specifically expressed in brain, and the promoter of this gene has two Krox-24-binding sites. Mutation of either Krox-24-binding site is sufficient to inactivate the gene, suggesting the Krox-24 factor may be involved in neuron-specific gene expression [35].

Mouse P19 cells, a line of pluripotent embryonic carcinoma stem cells, can be induced to differentiate into neurons and glia by treatment with retinoic acid. mGAD67 expression increases along neuronal differentiation of P19 cells [36]. It has been reported that there is an increase in Krox-24 transcript levels during P19 cell differentiation [34]. Moreover, previous studies with gel-shift assays showed that Krox-24-binding activity in the mouse neurofilament light-chain gene promoter is in good agreement with the time course pattern of the appearance of Krox-24 transcripts during P19 cell differentiation [37]. These results indicate that Krox-24 may participate in the regulation of mGAD67 gene expression.

Sp1 consensus elements bind a 100 kDa zinc-finger protein and confer basal activity on the promoters of viral or cellular genes [38–40]. Although originally associated with constitutive transcription, Sp1 can also co-operate with other transcription factors, such as NF-κB [41] and OTF-1 [42]. Figure 4(A) shows that an Sp1 site is close to the Krox-24 site in promoter A of the mGAD67 gene. It is tempting to speculate that co-operative interaction between Sp1 and Krox-24 might be involved in the regulation of the mGAD67 gene. Sp1 consensus motifs are found within the promoters of a number of neurally restricted genes [43,44], suggesting that the mGAD67 gene might be controlled by constitutive promoters whose activity is modulated by sequence-specific enhancer- and/or silencer-binding proteins to produce restricted patterns of expression. An F-ACT1-binding site was found in exon 0B (Figure 4B). F-ACT1 is a zinc-finger protein and is identical to a multifunctional transcription factor described as YY1 or NF-E1 [45]. It has been reported that four NF-E1 binding sites were present in the 5′-flanking region of an opioid receptor gene, KOR3, which is primarily expressed in brain [46]. Therefore, it is possible that F-ACT1 may be involved in brain-specific expression of exon 0B of the mGAD67 gene. The notion that these sequences might represent specific promoter motifs is being tested in transgenic mice.

Conclusions

The present characterization of the structural organization of the mouse GAD67 gene demonstrates the existence of multiple transcripts, which are generated by the alternative splicing of different 5′-exons on to a splice acceptor site within the 5′-UTR of the gene. The transcription of this gene thus appears to be under the control of alternative promoters that are activated in a tissue-specific manner which may play an important role in the regulation of the mGAD67 gene. The molecular and genetic mechanisms of phenotype selection of GABAergic synthesis and the maintenance of GABAergic function remain to be elucidated. Analysis of the expression mechanism of GAD genes will provide insights into the questions raised above. The assessment of GAD gene expression will await further studies in transgenic mice.

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