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Characterization of 5'- proximal sequence of mouse GABA transporter gene (GAT-1)[1]

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ABSTRACT

The cDNA molecule encoding the mouse GABA transporter gene (GAT-1) was used as probe for selecting GAT-1 gene from mouse genomic library. A positive clone, harboring the whole open reading frame of the GAT-1 protein and designated as MGABAT-G , was fished out from the library, the 5' proximal region and intron 1 were sequenced and analysed, and low homology was found in the above region between GAT-1 genes from mouse and human except some short conserved sequences. The DNA-protein interactions between DNA fragments containing the conserved sequences in the 5' proximal region and nuclear proteins from different tissues of mouse were studied by means of gel-shift assay, and Southern-Western blot. The results indicate a possible positive-negative regulation mode controlling the expression of the mouse GAT-1 gene.

Key words: *Mouse GABA transporter gene, expression regulation, cloning.*

INTRODUCTION

Sodium-dependent neurotransmitter transporters are membrane glycoproteins which were thought to constitute the neurotransmitter uptake systems in the brain[1, 2]. In the synaptic cleft, they are responsible for reuptaking neurotransmitters released from presynaptic neuron for terminating and resetting the synaptic signal

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transduction, and transporters existing in surrounding glial cells are also thought to play an important role in keeping regular concentration and distribution pattern of neurotransmitters in the nervous system. Besides, recent studies have revealed that neurotransmitter transporters can work in a reverse mode (releasing transmitters) and shows ligand-gated ion channel-like properties.

The complicated functions of neurotransmitter transporters are highly dependent on their special distributions in tissues, which are mainly determined by their gene expression regulation in the nervous system. So, the study of their gene expression regulation is important in understanding their functions in the nervous system.

γ-Aminobutyric acid (GABA) is the predominant inhibitory neurotransmitter in many parts of the vertebrate nervous system. Recently, four different GABA transporter subtypes (GAT1-4) with related sequences were cloned[3], and they show different affinities for GABA, different substrate and blocker pharmacologies, and different tissue localizations. Gat-1 is specially expressed in the central nervous system, the gene coding it from mouse and human was cloned by our lab (this paper) and Nelson's[4], but little is known about its gene expression regulation. In this paper, the interaction between nuclear proteins from different mouse tissues and 5'-proximal fragment of the mouse GAT-1 gene is reported. The results here provide helpful information for further study of the mechanism of GAT-1 gene expression regulation.

MATERIALS AND METHODS

Cloning of mouse GAT-1 gene

A mouse genomic library (Stratagene) cloned in pWE15 was screened by using a ^{32}p- labelled mouse GAT-1 cDNA fragment[5] (^{32}P-dATP, Amersham), and positive clones were picked up. The inserts were confirmed by Southern blot and sequence analysis (T7 Sequenceing Kit is from Pharmacia). Standard protocols were used for these experiments[6].

Gel shift analysis

Nuclear extracts from mouse (four weeks old, F1) brain, liver and kidney were prepared according to the method of the reference[7], protein concentration was assayed by the method of Lowry. DNA fragments for preparing probes were gel-purified after digestion by appropriate restriction enzymes (Promega) and ^{32}P labelled by fill-in using the Klenow enzyme (Promega). The probes were extracted with phenol/ chloroform and precipitated with ethanol. Gel shift analysis was done according to the description in the reference[8].

Southem- Western blot

Nuclear proteins from mouse brain, liver and kidney were separated by 10% PAGE, transferred to nitrocellulose membrane (Hybond C, Amersham), and then hybridization was done according to the reference[9].

RESULTS AND DISCUSSION

Several positive clones were fished out from the mouse genomic library by using mouse GAT-1 cDNA as a probe, and the one named MGABAT-G with an insert of

39kb in length contains the whole coding region of GAT-1 and 5'-proximal sequence accoording to the results of Southern blot and sequence analysis. The restriction physical map of MGABAT-G is presented in Fig 1, and the 5'-proximal sequence which includes exon 1 and exon 2 is shown in Fig 2.

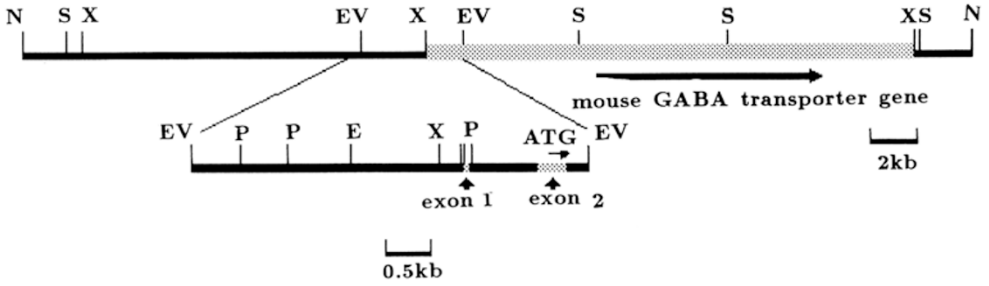

Fig 1. Physical map of the mouse genomic DNA coding for mouse GAT-1 gene (MGABAT-G)
N: Not 1; S: Sal I; X: Xho I; EV: EcoR V; P: Pst I; E: EcoR I

The transcription starting point of the gene was determined by using primer extension (data not shown), and it was found that the gene could be transcribed from multiple starting points and mainly were located at -1077, -1057, and -1054. There is no appropriate TATA box and CCAAT site which is usually 25-30bp uptream from the transcription site according to typical gene promoter structure. Instead, there are two short GC-rich region before the transcription site (-1206 ~ -1193, -1112 ~ -1097). TATA-box-less genes may use GC-rich or AT-rich region as their promoter[10], which can be found in some house-keeping genes and some tissure-specific genes, and in this case, genes tend to be transcribed from multiple starting points. A TATA-like sequence cluster is found in the 5'- proximal sequence, one is located starting from -1359, and the other three are found starting from -1411, -1477, -1531 respectively. Their precise functions (if they have) in gene transcription regulation await further study. It was found in some genes, such as the alpha-subunit of calcium/calmodulin-dependent protein kinase II, that remote TATA structures also have important roles in the promotion of gene transcription[11]. We also found in the first intron a TATA box sequence starting from -398, CCAAT sites at -423 and two potential transcription starting sites at -372 and -366, several SP1 sites and a cAMP- responsive elements (CREs) upstream of the TATA box, which together, all of these may form a potential promoter regulated by cAMP and the transcript from this promoter still codes the entire GAT-1 protein. Gomeza[12] reported that the expression of GAT-1 gene in neuron cell was down regulated by cAMP, but the details are still unknown, the information from the sequence analysis may help to clarify them.

Fig 2. 5' proximal sequence of mouse GAT-1 gene.
The translation starting codon(ATG) is located in exon 2, with A as position +1. Two conserved sequences are shown in italic characters and the exon region, Spl site, CCAAT sites, TATA-box sequence, CREs site and some restriction sites are marked in the sequence.

After a comparision of 5'-proximal sequence between mouse GAT-1 gene and human GAT-1 gene[13], low homology has been found in the above region, but there are two conserved sequences, one from -1664 to -1628 (37bp in length) and the other from -1405 to -1390 (16bp in length). In order to know if the 5'-proximal region which contains these conserved sequences functions in gene expression regulation, we cloned Sph I/ Xba I (containing the 37bp) and Xba I/Xho I (containing the 16bp) fragments (Fig 2). Gel shift analysis showed that there were several protein

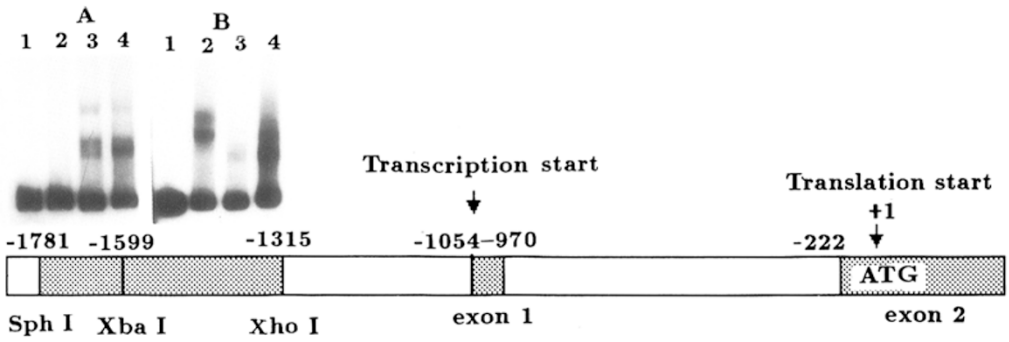

Fig 3. Gel-shift analysis of fragments containing 5'-proximal conserved sequences of mouse GAT-1 gene with nuclear proteins from different tissues. 2 ng (3000cpm, 50Bq) probe and 10 μg nuclear protein from different tissues were used in a total reaction volume of 20 μl, 2 μg poly dIdC was used as a non-specific competitor. After incubation at room temperature for 20 minutes, the reaction samples were run on 4% acrylamide gel.

A: Probes made from Sph I/Xba I fragment

1. Free probe as control. 2. Nuclear protein from brain. 3. Nuclear protein from liver. 4. Nuclear protein from kidney.

B: Probes made from Xba I/Xho I fragment

1. Free probe as control. 2. Nuclear protein from brain. 3. Nuclear protein from liver. 4. Nuclear protein from kidney.

factors from liver and kidney nuclear extracts which could specifically bind to the Sph I/Xba I fragment, but these protein factors are absent in the brain nuclear extract (Fig 3). Southern-Western blot also proved the existence of these proteins (there were at least three kinds of protein), and gave their molecular weights as 30KD, 28KD and 11KD respectively (Fig 4). The Xba I/Xho I fragment can be bound specifically by nuclear proteins from brain, liver and kidney, but at least one of the factors from brain was not present in those from liver and kidney (Fig 3). GAT-1 is a tissue- specific protein which is only expressed in brain. If the DNA-protein recognition above has biological functions, it is reasonable to consider that the interactions between Xba I/Xho I fragment and nuclear proteins from brain may play a positive role in the regulation of the gene expression and the proteins from

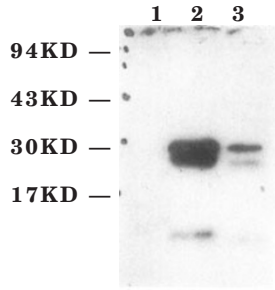

Fig 4. Southern-Western blot of nuclear protein from different tissues hybridized with Sph I/Xba I fragment of mouse GAT-1 gene. 20 μg of each nuclear proteins were run on 10% SDS-PAGE. The concentration of probe used was 1.8 Bq/ml.
1. Nuclear protein from brain, 2. Nuclear protein from liver, 3. Nuclear protein from kidney.

liver and kidney which can recognize Sph I/Xba I fragment may play a negative role in the regulation of the gene expression in liver and kindey. In other word, the Sph I/Xba I fragment may contain silencer sequence and sequence in Xba I/Xho I fragment may act as an enhancer for gene expression. The study of the transcriptional regulation of CNS-specific genes is of fundamental importance in understanding the differentiation, diversification, survival and plasticity of neurons. Recent studies have revealed that negative regulation plays a significant role in the control of neuron-specific gene expression[14]. The recently cloned silencer-binding factor NRSF/REST (neuron-restrictive silencer factor or REl-silencing transcription factor) is the first negative-acting transcription regulator to be implicated in vertebrate neuronal development and it functions in many neuron-specific genes[15, 16, 17]. In the 5'-proximal sequence of GAT-1 gene, we did not find the recognitive site for NRSF, so the tissue-specific expression pattern of the gene is determined by a way other than NRSF. The results described here indicate a possible positive-negative regulation mode controling of expression of the mouse GAT-1 gene. Promoter assay and mutagenesis experiment will provide us more information and cloning these nuclear proteins will be of great significance.

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