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Genomic Organization and Promoter Characterization of the Murine Glial Cell-derived Neurotrophic Factor Inducible Transcription Factor (mGIF) Gene

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To study the transcriptional mechanisms by which expression of the murine glial cell-derived neurotrophic factor inducible transcription factor (mGIF) gene is regulated, a murine genomic clone was isolated using a mGIF cDNA as probe. A 13-kb genomic fragment, which comprises 4-kb upstream of the transcription initiation site was sequenced. The promoter region lacks a TATA box and CAAT box, is rich in G+C content, and has multiple putative binding sites for the transcription factor Sp1. The mGIF gene also has consensus sequences for AP2 binding sites. The transcriptional activity of five deletion mutants of a 2.1-kb fragment was analyzed by modulating transcription of the heterologous luciferase gene in the promoterless plasmid pGL2-Basic. All mutants showed significant transcriptional activity in the murine neuroblastoma cell line NB41A3. Transient expression assays suggested the presence of a positive regulator between -213 and -129 while a negative regulator was found in the region between -806 and -214. Relatively strong transcriptional activity was observed in neuronal NB41A3, glial C6 cells and hepatic HepG2, but very weak activity in skeletal muscle C2C12 cells. These findings confirm the tissue-specific activity of the mGIF promoter and suggest that this gene shares structural and functional similarities with the dopamine receptor genes that it regulates.

Key words – Regulation, Sp1, TATA-less, transcription factor

Introduction

Gene expression in eukaryotes is controlled by a complex set of transcription factors that bind to distinct DNA elements. Especially, precise transcriptional regulation of the large number of genes is required for normal brain functions. Disorganizations in gene expression are associated with number of neuronal diseases. For instance, dopaminergic neurotransmission, which is critical in controlling cognition, movement and behavior, is altered in schizophrenia and Parkinson's disease [1,2]. Thus, elucidating the mechanisms that control the transcription level of genes involved in the control of dopamine mediated brain functions is necessary in understanding of the variables that regulate these genes in health and disease.

Detailed analysis of the transcription control mechanisms that regulate dopamine receptor genes has revealed complex patterns involving both activators and repressors [5,6,8-11]. The D_2 dopamine receptor gene which is highly expressed on the cell membrane of striatal neurons has a strong negative control element in its 5'-flanking region [11]. Screening a cDNA library from the D_2 expression neuroblastoma cell line NB41A3 with this negative control element as probe yielded a zinc finger type transcription factor, murine glial cell-derived neurotrophic factor (GDNF) inducible transcription factor (mGIF) [17]. mGIF was characterized a murine Sp1-related zinc finger transcription factor and is expressed in the adult and developing brain. This nuclear protein mGIF was induced the mRNA expression by GDNF [17].

In the present investigation, we characterized the genomic organization of the mGIF gene and analyzed its regulatory 5'-flanking region. In addition, cell type-specific transcriptional activity of this promoter was studied.

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Materials and Methods

Screening of Genomic Library and DNA Sequencing

A murine genomic library constructed in the bacterial artificial chromosome vector pBAC-Belo was screened with the murine mGIF cDNA [17] as probe. The 11-kb *Bam*HI-

*Eco*RI fragment, which was analysed by restriction analysis, Southern blots and complete sequencing, was found to represent the transcribed region and 4.0-kb 5′-flanking region of the mGIF gene. This *Bam*HI-*Eco*RI fragment was subcloned into pBluescript SK(+) (Strategene, Cedar Creek, TX, USA), yielding pBS-mGIF, for further characterization. Sequencing employed the Sanger dideoxynucleotide chain-termination method with Sequenase (United States Biochemical, Cleveland, OH, USA) using 7-deaza- dGTP [14].

5′-Rapid Amplification of cDNA Ends (RACE)

To determine transcription initiation site of the mGIF gene, 5′-RACE was performed with the 5′-RACE System for Rapid Amplification of cDNA Ends (Invitrogen Co., Carlsbad, CA, USA). First strand cDNA synthesis was performed using 1 μg poly(A)$^+$ RNA from mouse brain (Clontech Laboratories, Inc., Palo Alto, CA, USA) and 2.5 pmol of a gene-specific primer (5′-GAGGCTTGGCAGCAT CGGAG-3′ corresponding to nucleotides +499 to +480 relative to the guanosine at the transcription start site). After purification and dC-tailing of cDNA, primary polymerase chain reaction (PCR) was performed using a second gene-specific primer (5′-GCAGCATCGGAGAAAGATTTG AAGTGG-3′ +491 to +465) and an abridged anchor primer. PCR products were diluted and subjected to nested PCR using a third gene-specific primer (5′-GTAGATGGCGCTG ATGCAGTC-3′ +461 to +441) and an abridged universal amplification primer. Nested PCR products were subcloned in pGEM-T easy vector (Promega, Madison, WI, USA) and positive clones were confirmed by colony hybridization using the third gene-specific primer (nucleotides +461 to +441) as probe. Plasmid DNAs from positive clones were prepared and sequenced to determine transcription initiation site.

Construction of Plasmids

pBS-mGIF was digested with *Bam*HI and *Hin*dIII, and the 4.2-kb fragment that includes the 5′-flanking region was inserted into the corresponding sites of pUC19 yielding pUC-mGIF-4008/+248. pUC-mGIF-4008/+248 was digested with *Xba*I-*Nru*I and the 2.3-kb fragment was inserted into the *Xba*I-*Hin*cII sites of pUC19 yielding pUC-mGIF-2159/+93. pUC-mGIF-2159/+93 was digested with *Bgl*II and *Hin*dIII and the released fragment was inserted into the corresponding sites of pGL2-Basic (Promega) to yield pGL2-mGIF-2139/+93. The same strategy was employed to construct pGL2-mGIF-1161/+93, pGL2-mGIF-806/+93, pGL2-mGIF-213/+93 and pGL2-mGIF-128/+93 from pUC-mGIF-2159/+93. The integrity of all luciferase constructs was verified by restriction analysis and partial sequencing.

Cell Culture and Transient Expression Assays

The murine neuroblastoma NB41A3, rat glioma C6, human hepatoma HepG2, and mouse myoblast C2C12 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (BioWhittaker) at 37C in a humidified atmosphere containing 5% CO_2. Transfections were carried out using SuperFect Transfection Reagent (Qiagen, Inc., Valencia, CA, USA) with serum free DMEM in 60 mm dishes. A total of 3 μg of pGL2 plasmid and 2 μg of pCMVβ plasmid (Clontech) were used. The cells were harvested 48 hr later and lysed by adding 200 μg of 1× lysis reagent (a component of the Luciferase Assay System, Promega) to the harvested cells followed by centrifugation. All plasmids used in transfections were purified by the Plasmid Midi Kit (Qiagen). Luciferase assay was carried out using the Luciferase Assay System (Promega). All luciferase assay results were normalized to β-galactosidase activity [13].

Results

Nucleotide Sequence of the 5′-Flanking Region of the mGIF gene

To analyze the genomic structure of the mGIF gene, the 11-kb *Bam*HI-*Eco*RI fragment from a positive BAC clone was subcloned in pBluescript SK(+) to give plasmid pBS-mGIF (Fig. 1). The structure of pBS-mGIF was confirmed by restriction analysis and complete sequencing. Analysis of this clone revealed that the mGIF gene consists of 153 bp exon 1, 2704 bp intron 1, 231 bp exon 2, 94 bp intron 2, 913 bp exon3, 450 bp intron 3, and 1487 bp exon 4 (Fig. 1). This clone also includes 4.0-kb upstream of the transcription initiation site (Fig. 2). Sequence analysis of the 5′-flanking region revealed that the promoter lacks a TATA box and CAAT box (Fig. 2). This gene is relatively rich in G+C content, reaching 54% between nucleotides

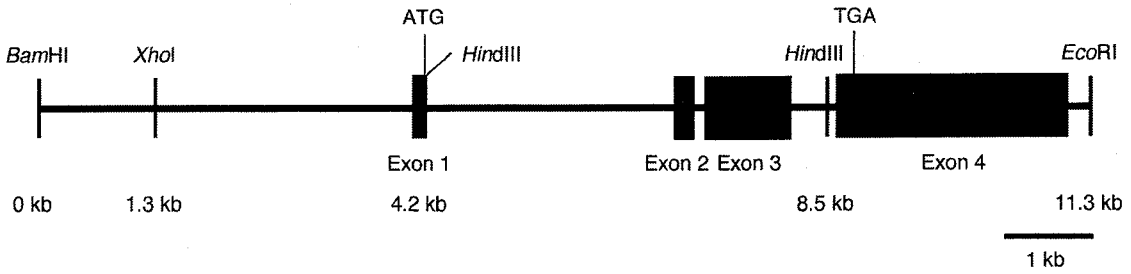

Fig. 1. Genomic structure of the mGIF gene. Exons are shown by black boxes. Intron and 5′-, 3′-UTRs are indicated by horizontal lines. Some restriction enzyme sites are shown.

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TCTAGAGCTGGGGACTAACTAGATCTCACTTAAGTTTTTGAGGGAGGGAGGCAACTGTGGCTAGACTGGC -2090
 XbaI        BglII
AATGATCACCCCTTAAGACCACAAAGAAAGAGGAGTTTTAGATTTGGCGATATTAGGTCTGAAGTGCCTG -2020
GGGAAGGCTTTCTCTGAATCTCCAGGTCACCACTACCCAGTTTCTCCTACCCCTTTCTCTCCTCTTTCCT -1950
                 ERE
GCTGTGATCACTCCAAGTCCCAAACGCTCCTTACAACTCAGCCTGCAGAGTGGGTGTGGGGGCACCACGC -1880
CAGACACAGGGTTCAGGCTTGGTGGGTCTGGCTGTGACCTCTCCCGGAGGCGGCTCCCTGACAGGAAGGC -1810
                          haTf ERE
AGGCAGGCAGGCCTGCCACTGGGCACACACAAGAGCTTCCTGCGCTGCAGCAGGTGCCCAGGCCAGCTGC -1740
   AP2                                                    AP2
TCCAGCCCTTCAGACTCAGATCAACAGGATGGGCCAGCCCAAGCGGAAGGAAGCCTGCAGGGAAGGACAC -1670
                              AP2
TCCTGCAGACAGAAGCAGGGCACCGGGTGGAGAGAGGCAGGAAGAGCTGCCTGGGTTGCTGAGTTGGCGC -1600
TCCTCCAGCGGATTCAGAGCAGGCAGTGACAGGAGGCCATTCAGTTATCTTTCCCCCACTTTCCACCCCC -1530
                                                        AP2
ACCCCAGGGCTTCTCTGGCCCACGTTATTATCATTACATTTGTTGCTGGCTGGCTTTAGGTATACCAGAA -1460
   AP2
GGAACCATCAGATAGATGTTGGTAGAGCTGGAGGCCTCTAAAACAAATTCCCTCCTTTACCTAGTCTGTG -1390
GTGACTGAGAAAGGGCACCTGCTTTAGCCCTTTTAGCCCAGCAGGCTGAAAAAGCCCACAGGAGGGCAGT -1320
GACTTTTGTCTCATGATCTGCCTGTCCCAGGGAGAAAATAAAAATCTCTAGTTTGCTTTAAGCCCCGTTA -1250
TCACTCCCTCCGGCTCCAACAGCTGCCGTGAACCCGCACAAACGGGCGAGGGGGCGGGGCAATAGGGCGC -1180
                                                  Sp1
CAACGAGGAGCCAAGAGCTCTCAGACTAGGAGGCTACAGCCTCAGCCGGGCTGCTGCACCCACAAGCTGG -1110
          SacI
CTTCCCTAAGAAGCTCAGCTTGCCCTCAATCCCTGGAGCGGTGGAGGGGGCAGGGACAGGAACTCGATGT -1040
TTGACCGGGTCAGGAATCCCACCTGCAAGCATGCTTTCCTCACCCCATCCCCTCAAGCGGTTTCTTGGTA  -970
   ERE                                  AP2
GGGCGTCGGCGAAGACTGTGGTCCCGCGGGTGGGGGAAGTGGAGTCCACCTTGGAAATAGCTCGAACATT  -900
GTATTTTAAACTTCAACTACATCAAACTCAAACTCGATTCTGCCACCGAGCGTCGGGTCTGGGGCTCCCT  -830
GCCACGCCACTAGCTCTTTGGTACCGGCTTCCCTTAGTCCCCACTCCAACCGCCCAGAAGGACGGGAGGG  -760
                    KpnI
ACGCGCAGAGGCGGGCCCTGCTGCTAGTGCCTGTGCTGCAGATGGGAGGACGAGCCAGCGCCTGCCGCCT  -690
TGGGCGTGGGCGGTGAGTGCGAGCAGGTGTCTGTTCCGTGACAGGGCTCCCGCCGCCACCATCTCGCCCT  -620
  Sp1
GGTTCTCCCCAGTCCTCCGGCTTAGAGTGACAAAACCCGGCAAATGCTCAGAAATGCCGCTCAGTGGTTC  -550
ATCCATCCCTTGCTTCCAGGCAAGACCCACGAGAACCACGCTGCCAAAGAACGTCTGGTGGCTCGGAGCC  -480
GCGCTCGCAGCCTCCGCCTCCGCCTCCTGCTCGGGGTCCCGCCCACTCCCTGGCGGGCCCAGACGCTGTG  -410
GGCGCGAAGGGGACTGGGCGGAGCGGGCTTCCCTCCCCACCCCCCACGTGGGGCCGGCTCTCCGCAAAGC  -340
                Sp1                 AP2
CTAGTCGCCGCCACCGCGGCCTCCCAGCTATGCCCCGCCGCGGCGCCGCCCCCGGGCATGGGCGGAGGAC  -270
   AP2                 AP2                 AP2 SmaI    Sp1
TGAAGGCTAGGGTTGGGCGAGGGGCGTCACGGAGTCAGGTGGTCCCCGGAGTTCCCCGGGGCTGGACCGA  -200
                                                AP2 SmaI
GGGAACACGGTGCCTCGGGTTGTGTACACGCTCCACTGACAGAGCCTCTTGCAGCCGGGCAGCCGCTGAT  -130
CACGCGTGGCTCTGCCAGCTCATTGGCTGAGGCCTCACACACCTTTGCCGCTGATTGGTCGACACTAGGC   -60
 MluI                          Pro/Gro
CCCGCCCTCTACCCCGCCCGCGGCGCGGGGCAGGCAGAGCGGCTACCTGCACGGGGAGGGGGGCAGACAG   +11
Sp1      Sp1  AP2
CGCGGAGCGCGGTGGCGTCGACGTCTAGTGTCTCAGTGCTCCCGTCTGTGGCTAACTAAGCAGCCAGCAG   +81
CCAGGCAGCTCGCGACCTGCGGCCAGGCAGCCAACCATGCTCAACTTCGGCGCTTCTCTCCAGCAAGCTT  +151
    NruI                          M  L  N  F  G  A  S  L  Q  Q  A          +11
CGgtaagttacctgaggatgctggtcctttcctgccggtcagtgtaggggagccagccggagcgcgaagc  +221
S                                                                         +12
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Fig. 2. Nucleotide sequence of the 5′-flanking region of the mGIF gene. The sequence is numbered with an guanosine (double underlined) at the transcription start site as +1. The 5′ end of the intron is shown in lowercase letters. Putative transcription factor binding sites are underlined. Some restriction enzyme sites used to generate luciferase constructs are shown.

-4011 and +93, 62% between -2159 and +93, and 69% between -128 and +93. Homology analysis revealed consensus sequences for several transcription factor binding sites including Sp1 [4] at positions -48, -60, -280, -393, -682, and -1200 and AP2 [12] at positions -43, -219, -294, -308, -332, -376, -996, -1528, -1534, -1707, -1753, and -1804 (Fig. 2). The functional significance of these consensus sequences in the expression of the mGIF gene remains to be determined. These features of the mGIF gene, namely transcription from a TATA-less promoter, high G+C content and multiple potential Sp1 binding sites are also seen in housekeeping genes [4].

Determination of the Transcription Start Site of the mGIF gene

Transcription initiation site was determined by 5'-RACE. To eliminate non-specific amplification of the first PCR, nested PCR was performed. Colony hybridization was also performed to exclude non-specific clones. All two specific clones thus generated had an insert size of 461 bp and ended at the same guanosine at their 5'-ends. Additional rounds of 5'-RACE did not uncover upstream cDNA sequences.

Transcriptional Activity of the 5'-Flanking Region of the mGIF Gene

To determine the regulatory regions in the 5'-flanking region of the mGIF gene, five restriction fragments were subcloned into pGL2-Basic, yielding five serial deletion mutants (Fig. 3 and 4). All constructs had transcriptional activity in NB41A3 cells, which are known to express both mGIF and the D_2 dopamine receptor [17] (Fig. 3). pGL2-mGIF-213/+93, which contains 306 bp *Sma*I-*Nru*I fragment of the mGIF gene showed the strongest transcriptional activity reaching 106 fold compared with pGL2-Basic. pGL2-mGIF-2139/+93, which contains 2286 bp *Bgl*II-*Nru*I fragment of the mGIF gene showed relatively strong transcriptional activity reaching 59 fold compared with pGL2-Basic. On the other hand, pGL2-mGIF-1161/+93, which includes the 1254 bp *Sac*I-*Nru*I region of the mGIF gene, had the weakest transcriptional activity reaching 40 fold compared with pGL2-Basic. pGL2-mGIF-806/+93 and pGL2-mGIF-128/+93 showed moderate transcriptional activity reaching 48 and 57 fold, respectively, compared with pGL2-Basic. pGL2-mGIF-213/+93, which contains the 306 bp *Sma*I-*Nru*I fragment showed about 2.2-fold higher activity than pGL2-mGIF-806/+93, suggesting that the 594 bp *Kpn*I-*Sma*I region contains negative regulatory element(s). Further deletion in the 5' end of the mGIF gene from pGL2-mGIF-213/+93 (pGL2-mGIF-128/+93) resulted in significant decrease in activity reaching 54% of that of pGL2-mGIF-213/+93, suggesting the presence of positive regulatory element(s) between -213 and -129. The 221 bp fragment of pGL2-mGIF-128/+93, which contains con-

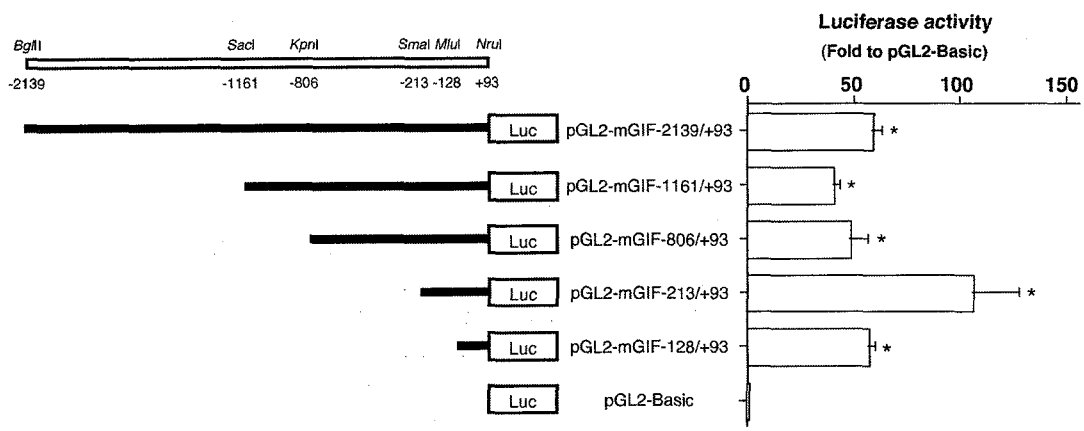

Fig. 3. Transient expression analysis of the 5'-flanking region of the mGIF gene in murine neuroblastoma NB41A3 cells. Schematic representation of luciferase constructs containing various lengths of the mGIF 5'-flanking region is shown on the left. Restriction sites used for subcloning are shown. Each luciferase construct was used to co-transfect NB41A3 cells along with pCMV as internal control. Luciferase activity was measured using the Luciferase Assay System, normalized with β -galactosidase activity derived from pCMV and expressed as fold increase over the promoterless reporter plasmid pGL2-Basic. Results presented are the means SEM for three plates. Factorial ANOVA with Fisher's PLSD post-hoc test *p<0.0004 compared with pGL2-Basic. Transfections were repeated at least twice yielding reproducible results.

sensus sequences for two Sp1 binding sites, was still able to promote transcriptional activity (Fig. 2 and 3). This observation suggests that these putative Sp1 binding sites could activate basal promoter activity. Furthermore, because mGIF binds the Sp1 binding site of the D2 gene [17], it is possible that mGIF itself regulates the gene transcription of the mGIF gene through Sp1 binding sites of this gene.

Cell Type-Specific Transcriptional Activity of the 5′-Flanking Region of the mGIF Gene

To analyze the cell type-specificity of the mGIF promoter, five serial deletion mutants were used to transfect three different cell types (Fig. 4). All deletion mutants had transcriptional activity in both glial C6 and liver (HepG2) cells. pGL2-mGIF-2139/+93 showed the strongest transcriptional activity in neuronal NB41A3 cells compared with glial C6 and hepatic HepG2 cells (Fig. 3 and 4). However, pGL2-mGIF-1161/+93, pGL2-mGIF-213/+93 and pGL2-mGIF-128/+93 showed the strongest transcriptional activity in HepG2 cells, compared with other cells tested (Fig. 3 and 4). On the other hand, pGL2-mGIF-806/+93 showed the strongest transcriptional activity in C6 cells, compared with other cells tested (Fig. 3 and 4). However, All deletion mutants showed very weak transcriptional activities in skeletal muscle C2C12 cells (Fig. 4). These results from transient expression analyses are consistent with the tissue specific distribution pattern of mGIF mRNA [17].

Discussion

In the present report, we analyzed the genomic structure of the mGIF gene with its 5′-flanking region, and characterized its promoter by means of transient expression assays. The mGIF gene has its coding sequences distributed over four exons (Fig. 1). Transcription in this gene begins at a single guanosine with no canonical TATA or CAAT boxes in the immediate upstream sequence, although TATA-less promoters often tend to have multiple transcription start sites [4]. Analysis of the 5′-flanking region revealed several consensus sequences for Sp1 binding sites at positions -48, -60, -280, -393, -682, and -1200 (Fig. 2). This gene is rich in G+C content, achieving 70% in some portions. These structural features are seen in housekeeping genes, which are expressed at low levels in essentially all tissues [4]. However, expression pattern of the mGIF gene has a high degree of tissue specificity with its mRNA detected mainly in brain but not in skeletal muscle

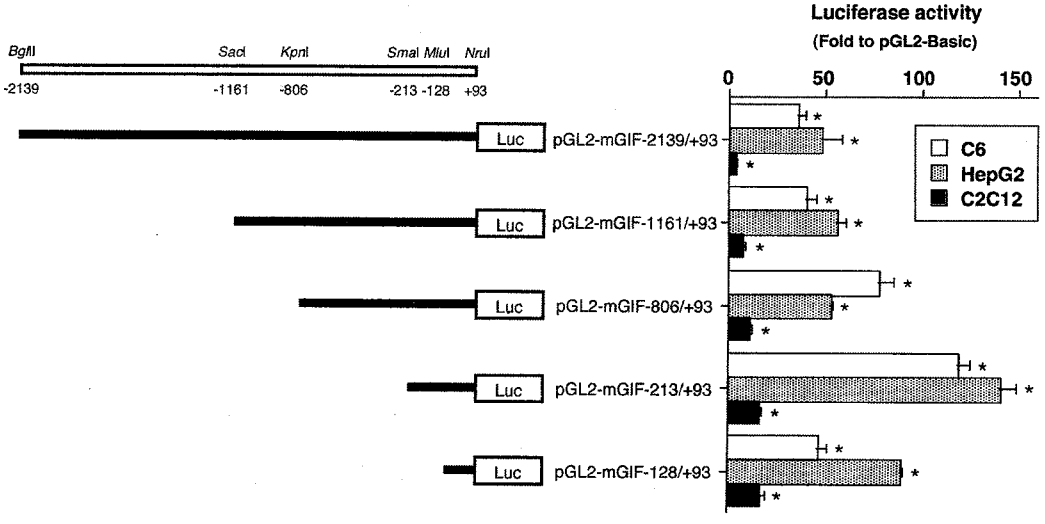

Fig. 4. Transcription analysis of the 5′-flanking region of the mGIF gene in different cell types. Schematic representation of luciferase constructs containing various lengths of the mGIF 5′-flanking region is shown on the left. Restriction sites used for subcloning are shown. Each luciferase construct was used to co-transfect rat glioma C6, human hepatoma HepG2, and murine myoblast C2C12 cells along with pCMV as internal control plasmid. Luciferase activity was measured using the Luciferase Assay System, normalized with β-galactosidase activity derived from pCMV and expressed as fold increase over pGL2-Basic. Results presented are the means SEM for three plates. Factorial ANOVA with Fisher's PLSD post-hoc test * $p<0.0001$ compared with pGL2-Basic. Transfections were repeated at least twice yielding reproducible results.

[17]. In fact, a large number of tissue-specific genes, including some expressed only in neural cells, have similar housekeeping-type promoters, such as the D_{1A} [8] and D_2 [10] dopamine receptor genes, dopamine receptor regulating factor gene [7], nerve growth factor receptor gene [16] and synapsin I gene [15].

Transcriptional analysis revealed that the mGIF gene has both positive and negative control elements, needed to tightly regulate its levels and hence dopaminergic neurotransmission. Putative transcription factor binding sites in the negative region (nucleotides -806 to -214) includes Sp1 and AP2 (Fig. 2 and 3). However, no putative transcription factor binding site was found in the positive region (nucleotide -213 to -129) (Fig. 2 and 3). These regions remain to be investigated.

We had previously demonstrated that the mGIF mRNA is expressed abundantly in brain and liver, but not in skeletal muscle [17]. Consistent with this tissue specific distribution, we observed relatively strong transcriptional activity of the mGIF promoter in neuronal NB41A3, glial C6 and hepatic HepG2 cells, but very weak activity in skeletal muscle C2C12 cells (Fig. 3 and 4).

We conclude that mGIF is transcribed from a tissue-specific promoter that is tightly regulated transcriptionally, as expected from a transcription factor whose function appears critical in maintaining a delicate balance of genes transmitting dopaminergic signals and likely other as yet unidentified target genes.

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초록 : 생쥐 신경교세포 유래 신경영양인자 유도성 전사인자 (mGIF) 유전자의 유전체 구조 및 프로모터 특성 분석

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생쥐 신경교세포 유래 신경영양인자 유도성 전사인자(mGIF)의 발현조절에 필요한 전사기작을 연구하기 위하여 mGIF cDNA를 탐침자로 이용하여 genomic clone을 분리하였다. 전체 유전자 13-kb 영역 중 전사개시점에서 4-kb 상류영역의 유전자 서열을 파악한 결과, 프로모터 영역에서 TATA box와 CAAT box는 발견할 수 없었으며 G+C content는 높은 것으로 나타났고 여러 개의 Sp1 전사인자 결합영역이 있었다. 또한 mGIF 유전자는 AP2 결합에 필요한 보존적 영역이 있었다. mGIF 유전자의 프로모터 영역의 단편들을 프로모터가 없는 pGL2-Basic 플라스미드의 luciferase 유전자의 상류에 연결하여 서로 다른 5 종류의 결손 돌연변이체를 제조하고 NB41A3 세포주를 이용하여 전사활성을 측정하였다. Transient expression assays 결과, 모든 결손 돌연변이체에서 전사활성이 나타났으며 -213과 -129 사이에 전사촉진 영역이 존재하며 -806과 -214 사이에 전사억제 영역이 있는 것으로 나타났다. 신경세포주인 NB41A3과 신경교세포주인 C6 그리고 간세포주인 HepG2에서 mGIF 유전자 프로모터의 높은 활성이 관찰되었으며, 근육세포주인 C2C12에서는 낮은 활성이 관찰되었다. 따라서 mGIF 유전자는 조직특이적으로 발현하며 도파민 수용체 유전자와 구조적, 기능적 유사성이 있는 것을 알 수 있었다.