Acta Biochim Biophys Sin (2009): 237–245 | © The Author 2009. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmp007.

Isolation and functional analysis of the human glioblastoma-specific promoter region of the human GD3 synthase (*hST8Sia I*) gene

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We identified the promoter region of the human GD3 synthase (*hST8Sia I*) gene to elucidate the mechanism underlying the regulation of *hST8Sia I* expression in human glioblastoma cells. The 5′-rapid amplification of cDNA end using mRNA prepared from U-87MG cells revealed the presence of transcription start site of *hST8Sia I* gene, and the 5′-terminal analysis of its product showed that transcription started from 648 nucleotides upstream of the translational initiation site. Functional analysis of the 5′-flanking region of the *hST8Sia I* gene by transient expression method revealed that the region from −638 to −498 is important for transcriptional activity of the *hST8Sia I* gene in U-87MG and T98G cells. This region lacks apparent TATA and CAAT boxes, but contains putative binding sites for transcription factors AREB6 and Elk-1. Site-directed mutagenesis and transient transfection assays demonstrated that both AREB6 and Elk-1 elements in this region were required for the promoter activity in U-87MG and T98G cells. These results indicated that both AREB6 and Elk-1 might play an essential role in the transcriptional activity of *hST8Sia I* gene essential for GD3 synthesis in human glioblastoma cells.

Keywords promoter; human GD3 synthase (*hST8Sia I*) gene; glioblastoma; transcription factor

Received: October 20, 2008 Accepted: November 25, 2008

Introduction

Gangliosides are the sialic acid (NeuAc)-containing glycosphingolipids, which are present in the outer leaflet of the plasma membrane of vertebrate cells and are particularly abundant in the central nervous system [1]. They play important roles in a large variety of biological processes, such as cell–cell interaction, adhesion, cell differentiation, growth control and receptor function [2]. Many previous studies showed that aberrant ganglioside expression was strongly associated with the malignancy of tumor cells and that tumor-associated gangliosides played a crucial role in the induction of tumor cell invasion and metastasis [3,4]. It was also known that the composition and the content of gangliosides changed during physiological growth and differentiation as well as in neoplastic cell transformation [5–9]. In addition, changes in the composition and the content of gangliosides have been observed in human gliomas, which are the most common form of human primary brain tumor [9–11].

Among the various gangliosides, GD3 is a structurally simple ganglioside and is related to malignant transformation. Previous studies have demonstrated that malignant gliomas contain a higher concentration of GD3, and its expression correlates with the degree of malignancy [8,9]. GD3 is synthesized by GD3 synthase (ST8Sia I; EC 2.4.99.8), which is a key enzyme for the synthesis of whole b-series gangliosides including GD2 in addition to GD3 itself [12]. It is known that GD3 expression generally appears to be regulated at the transcriptional level of *ST8Sia I* gene [13,14]. In order to understand transcriptional regulation mechanism for *ST8Sia I* gene expression in malignant glioblastoma cells, it is very important to characterize the promoter function of *GD3* synthase gene. Although the increased expression of human GD3 synthase (*hST8Sia I*) mRNA with

a concomitant increase of GD3 has been recently reported [15], the transcriptional regulation mechanism for glioblastoma-specific expression of *hST8Sia I* gene has not yet been studied.

In this study, the promoter region to direct up-regulation of *hST8Sia I* gene transcription in human glioblastoma cells was functionally characterized. The present results indicated that both AREB6 and E1 k-1 binding sites of the hST8Sia I promoter played a critical role in the transcriptional regulation of *hST8Sia I* expression necessary for GD3 synthesis, which is highly expressed in human glioblastoma cells.

Materials and Methods

Cell cultures

Human neuroblastoma cell line SK-N-BE(2)-C, human breast carcinoma cell line MCF-7 and human glioblastoma cell lines U-87MG and T98G were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). These cell lines were grown in Dulbecco's modified Eagle's medium (WelGENE Co., Daegu, Korea) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C under 5% CO_2. Human Jurkat T cells from ATCC were cultured in RPMI 1640 supplemented with 1 mM sodium pyruvate and 1× MEM non-essential amino acids.

Evaluation of ganglioside GD3 surface expression

Analysis of ganglioside GD3 levels on the cell membrane was carried out as described previously [16]. Briefly, SK-N-BE(2)-C, U-87MG and T98G cells were seeded on round cover slips (pretreated with 2% of 3-aminopropyl-triethoxysilan) in an eight-well multiplate. After incubation with the GD3 monoclonal antibody (mouse IgM, Kappa-chain, clone, GMR19; Seigakagu, Tokyo, Japan) diluted 1:500 with 5% bovine serum albumin in phosphate-buffered saline, cells were labeled with 1:500 FITC-conjugated goat anti-mouse IgM (Sigma, St Louis, MO, USA) as a secondary antibody. The samples were mounted with glycerol and analyzed by Confocal laser-scanning microscope (LSM 510; Zeiss, Jena, Germany).

Reverse transcription-polymerase chain reaction and 5′-rapid amplification of cDNA end

Total RNAs isolated from cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) were subjected to reverse transcription with oligo d(T) using RNA polymerase chain reaction (PCR) kit (TaKaRa, Tokyo, Japan) according to the manufacturer's protocol. The cDNA of *hST8Sia I* and β-actin as a control was amplified with the following primers: *hST8Sia I* (460 bp), 5′-TGTGGTCCAGAAAGACATTTGTGGACA-3′ (sense) and 5′-TGGAGTGAGGTATCTTCACATGGGTCC-3′ (antisense); β-actin (247 bp), 5′-CAAGAGATGGCCAC GGCTGCT-3′ (sense) and 5′-TCCTTCTGCATCCTGT CGGCA-3′ (antisense). PCR amplification was performed using the following conditions: 94°C for 2 min and then 35 cycles of 94°C for 40 s, 60°C for 45 s and 72°C for 40 s, with a final elongation of 72°C for 10 min. The PCR products were separated by gel electrophoresis on 1% agarose containing ethidium bromide. To assess the specificity of the amplification, the PCR product for GD3 synthase was subcloned into pGEM-T vector (Promega, Madison, WI, USA) and then sequenced. Amplification of the 5′-terminal of GD3 synthase was performed with the 5′-rapid amplification of cDNA end (5′-RACE) kit (Invitrogen) according to the manufacturer's instructions, using 5 μg of mRNAs from U-87MG cells. The gene-specific primer GD3RT [17] was used for initial reverse transcription. After synthesis of the first strand cDNA, an Abridged anchor primer provided by the company and the gene-specific primer GSP1 [17] were used in the first PCR. The second PCR was performed at 65°C using an Abridged universal amplification primer and the gene-specific primer GSP2 (5′-CGCGGGAACTTCC ACGCCAGTACAGC-3′, complementary to nucleotides 44–70). The PCR products were subcloned into pGEM-T vector and sequenced.

Quantitative real-time polymerase chain reaction

Total cellular RNA and single-stranded cDNA were prepared from cells as described above. Real-time PCR was performed on a Rotor Gene 3000 (Corbett Research) with the QuantiTect® SYBR® Green PCR Master Mix (Qiagen, Hilden, Germany). Samples were standardized to the β-actin mRNA level. The transcripts of the *hST8Sia I gene* were detected by the quantitative real-time PCR approach using the primers 5′-TTCAACCTCT CTCTTCCCACA-3′ (sense) and 5′-TCTTCTTCAGAAT CCCACCATT-3′ (antisense) from *hST8Sia I* sequences (GenBank accession no. D26360.1). The transcript copy number of the *hST8Sia I* gene was normalized to the β-actin transcript copy number for each sample. As a standard control, the β-actin was amplified by the primers 5′-ACCCACTCCTCCACCTTTGAC-3′ (sense) and 5′-CCTGTTGCTGTAGCCAAATTCG-3′ (antisense)

Table 1 Primer sequences used in this study

Primer	Sequence	Strand	Position
P-998S	5′-GG*GAGCTC*AAGGTACCAGACACCGCAG-3′	Sense	−998 to −979
P-818A	5′-AT*CTCGAG*GGCCGACGCATAGCTCG-3′	Antisense	−836 to −818
P-818S	5′-GG*GAGCTC*GCCGGGGCGAGCAGAAAT-3′	Sense	−818 to −800
P-638A	5′-AT*CTCGAG*ATTGCCTTGGAGGCGC-3′	Antisense	−654 to −638
P-638S	5′-GG*GAGCTC*GTGACATCTTGAGGT-3′	Sense	−638 to −623
P-498A	5′-AT*CTCGAG*GTTCACGGGCGCAAGCG-3′	Antisense	−515 to −498
MuElk-1-s	5′-CTGGAT*GCAGCA**GAGCTC**TTCAG*GGAGGAGGCGAGGCGG-3″	Sense	−593 to −554
MuElk-1-a	5′-CTCC*CTGAA**GAGCTC**TGCTG*CATCCAGATGTGCAGGCAGCTGG-3′	Antisense	−609 to −566
MuAREB6-s	5′-GAGTGC*ACG**GAGCTC**CCTC*GTGCGCGCGCTCGCTTG-3′	Sense	−545 to −510
MuAREB6-a	5′-CGCAC*GAGG**GAGCTC**CGT*GCACTCCGGGGTCCTCCG-3′	Antisense	−557 to −522

Primers P-998S to P-498A were used for construction of the deletion mutants. These contain *Sac*I and *Xho*I sites in sense and antisense primers, respectively. The mutated nucleotides in the oligonucleotides for mutation are represented in bold and italics. The primers used for site-directed mutagenesis include the restriction enzyme *Sac*I site (GAGCTC). The number indicates the nucleotide positions relative to transcription start site (+1) of *hST8Sia I* gene.

from human β-actin (GenBank accession no. NM_001101.3). Real-time PCR amplification of the *hST8Sia I* and β-actin genes was carried out for 50 cycles of 94°C for 10 s, 58°C for 15 s, and 72°C for 15 s.

Preparation of reporter plasmids and mutagenesis

The luciferase reporter plasmid, pGL3-1998 and its derivatives (pGL3-498 to pGL3-1598) have been described elsewhere [17]. Other reporter plasmids (pGL3-998/-818, pGL3-818/-638 and pGL3-638/-498) were generated by PCR with sense and antisense primers containing *Sac*I and *Xho*I sites, respectively (**Table 1**), using pGL3-998 [17] described previously as template. The PCR fragments were subcloned into pGEM-T Easy vector (Promega) and sequenced. Each fragment obtained by digestion with *Sac*I and *Xho*I was inserted into the corresponding sites of the pGL3-Basic vector, which was used as a negative control. Mutants with base substitution at the Elk-1 and AREB6 binding sites were constructed using a QuikChange® II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol using the following oligonucleotide primers as shown in **Table 1**. The presence of mutation was verified by sequence analysis.

Transfection and luciferase assay

To analyze *hST8SiaI* promoter activity, 1×10^6 cells/well were seeded in 24-well tissue culture plates and allowed to grow to 70% confluence, at which point they were transiently co-transfected with 0.5 μg of the indicated reporter plasmid and 50 ng of the control Renilla luciferase vector pRL-TK (Promega), using 1 μl of Lipofectamine 2000 (Invitrogen). After a 12-h recovery in normal medium, cells were collected and treated with passive lysis buffer (Promega). Firefly and Renilla luciferase activities were measured using the dual-luciferase reporter assay system (Promega), according to the manufacturer's instructions, and a GloMax™ 20/20 luminometer (Promega). Firefly luciferase activity of the reporter plasmid was normalized to Renilla luciferase activity and expressed as a fold induction over the empty pGL3-Basic vector, used as a negative control. Independent triplicate experiments were performed for each plasmid.

Results

Expression of *hST8Sia I* gene and ganglioside GD3 in human glioblastoma cells

To investigate expression of *hST8Sia I* gene involved in GD3 production, we analyzed the expression profile of *hST8Sia I* gene in human glioblastoma cells. As shown in **Fig. 1**, Reverse transcriptase (RT)-PCR and quantitative real-time PCR showed that an expression of *hST8Sia I* mRNA became detectable in human glioblastoma U-87MG and T98G cells only. The level of expression was high in these cells, while it was at very low levels in human neuroblastoma SK-N-BE(2)-C cells, human breast carcinoma MCF-7 cells and human Jurkat T-cells.

To investigate whether the expression levels of *hST8Sia I* mRNA correlate with cellular levels of the ganglioside GD3 in human glioblastoma cells, we used immunofluorescent Confocal microscopy to visualize

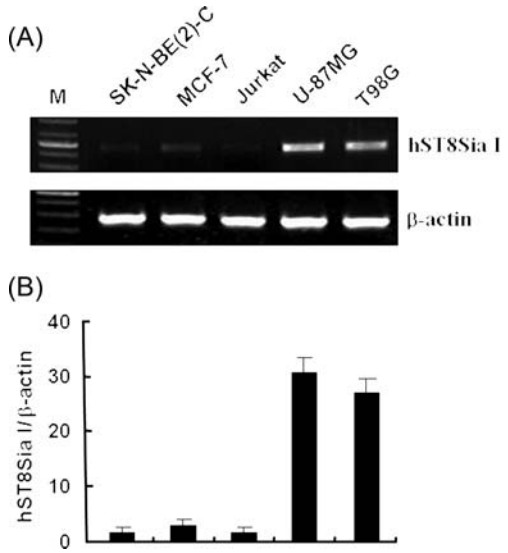

Fig. 1 Expression of hST8Sia I mRNA from human cancer cells (A) Total RNAs from SK-N-BE(2)-C, MCF-7, Jurkat T, U87MG and T98G cells were isolated and *hST8Sia I* mRNA was detected by reverse transcriptase-polymerase chain reaction (PCR). As an internal control, parallel reactions were performed to measure the levels of the housekeeping gene β-actin. (B) *hST8Sia I* mRNA expression was analyzed by quantitative real-time PCR. The transcript copy number of *hST8Sia I* was normalized to the β-actin transcript copy number for each sample. Experiments were repeated five times to ascertain reproducibility of results. The error bar indicates standard error.

GD3 expression in cells. We observed a higher GD3 expression in human glioblastoma U-87MG and T98G cells than in human neuroblastoma SK-N-BE(2)-C cells showing low level of *hST8Sia I* expression (**Fig. 2**).

Identification of the transcription start site and sequence analysis of the 5′-flanking region of *hST8Sia I* gene

As the first step necessary to analyze the glioblastoma-specific promoter activity of 5′-flanking region of *hST8Sia I* gene, we tried to determine the transcription start site of the *hST8Sia I* gene from mRNA prepared from U-87MG cells by the same 5′-RACE method as we reported previously [17,18]. When the secondary nested PCR was performed according to the manufacturer's instructions, the PCR product of about 0.7 kb fragment was obtained [**Fig. 3(A)**]. From the sequence analysis of this product, the transcription start site of the *hST8Sia I* in U-87MG cells was found at 648 bp upstream of the ATG codon [**Fig. 3(B)**]. This sequence included a 482 bp segment at its 3′-terminal that exactly matched the sequence in the 5′-untranslated region of the previously reported *hST8Sia I* cDNA [14], as described previously [17,18]. Based on this finding, we identified the promoter region of the *hST8Sia I* gene. As shown in the previous reports [17,18], the sequence analysis revealed that this region lacks canonical TATA and CAAT boxes but contains several putative transcription factor-binding sites including c-Myb, Elk-1, AREB6, GATA-1, c-Ets-1, cAMP-responsive element-binding protein, activating protein-1, and NF-κB.

Analysis of transcription activity of 5′-flanking region of *hST8Sia I* gene

To analyze whether or not the 5′-flanking sequence of the *hST8Sia I* gene contains a glioblastoma-specific promoter, we used reporter plasmid, pGL3-1998, in which about 2 kb upstream of the putative transcription start site of the *hST8Sia I* gene was fused to the promoter-less and enhancer-less luciferase gene in pGL3-Basic. This reporter plasmid and pGL3-Basic plasmid as a negative control were transfected into the human glioblastoma U-87MG and T98G cells and human neuroblastoma SK-N-BE(2)-C cells, and changes in the promoter activities were analyzed. As shown in **Fig. 4(A)**, the pGL3-1998 showed a remarkable increase of promoter activity in U-87MG and T98G cells, which was about 3-fold higher than in SK-N-BE(2)-C cells. This result clearly suggests that the region between nucleotides −1998 and +1 was important for the endogenous expression of *hST8Sia I* gene in U-87MG and T98G cells.

Based on this finding, to determine the minimal promoter region controlling the maximal promoter activity of the *hST8Sia I* in U-87MG and T98G cells, we prepared four additional reporter plasmids (pGL3-498 to pGL3-1598) containing progressive 5′ deletions and transfected into U-87MG and T98G cells and SK-N-BE(2)-C cells as a negative control, and changes of the promoter activities in U-87MG and T98G cells when compared with SK-N-BE(2)-C cells were analyzed. As shown in **Fig. 4(A)**, the progressive removal of fragments stretching between nucleotide −1998 and −1308 showed a similar activity with pGL3-1998. However, deletion in the region from −1308 to −998 resulted in increase of transcription activity, while deletion in the region from −998 to −498 markedly reduced transcription activity to the level of the pGL3-1998. These results suggest that potential positive regulatory elements exist within the region from −998 to −498, while potential negative regulatory elements exist within the −1308 to −998. These results also suggest that the region between −998 and −498

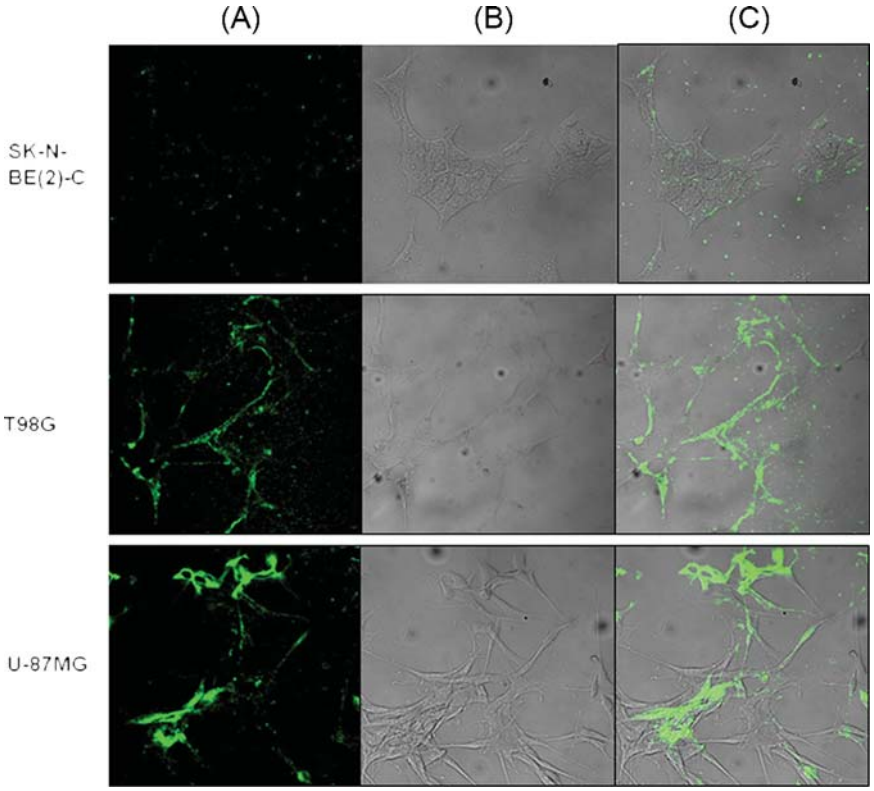

Fig. 2 Confocal analysis of ganglioside GD3 levels SK-N-BE(2)-C, T98G and U-87 cells were grown in standard medium. A monoclonal antibody (GMR19) was used to analyze the resulting GD3 ganglioside levels: (A) immunofluorescence image; (B) phase-contrast image; (C) merge.

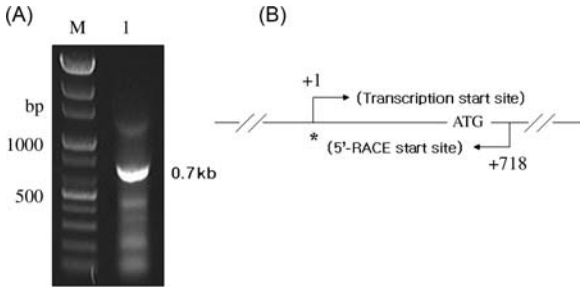

Fig. 3 Identification of the transcription start site in the 5′-flanking region of *hST8Sia I* gene by 5′-rapid amplification of cDNA end (5′-RACE)–polymerase chain reaction Total RNA from U-87MG cells was prepared. (A) Reverse transcription reaction was performed using GD3RT primer and PCR was performed with Abridged anchor primer and GSP1 primer with 5′-RACE strategy. The resulting product (lane 1) was analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. (B) Transcription initiation sites of *hST8Sia I* gene in U-87MG cells. The star indication represents the transcription start site as resulted in 5′-RACE. The number indicates the nucleotide positions relative to transcription start site (+1) located at 648 bp upstream of the ATG codon.

probably functions as the core promoter essential for transcriptional activation of *hST8Sia I* in U-87MG and T98G cells.

Identification of the core promoter essential for transcriptional activation of *hST8Sia I* gene in U-87MG cells

Based on this result, to determine further whether the region from nucleotides −998 to −498 has key elements essential for transcriptional activation of *hST8Sia I* gene in human glioblastoma cells, we also prepared three additional reporter plasmids containing simultaneous deletions from both 5′-terminal and 3′-terminal of the *hST8Sia I* gene promoters. After transfection of these plasmids into U-87MG and SK-N-BE(2)-C cells, promoter activity of the *hST8Sia I* gene was determined. As shown in **Fig. 4(B)**, the progressive removal of fragments stretching between nucleotide −998 and −638 resulted in a gradual increase in transcriptional activity, suggesting that this region contains negative regulatory elements for transcription. The maximum activity was obtained with pGL3-638/-498 and reached to 4-fold higher activity than SK-N-BE(2)-C cells. These results show that the region between nucleotides −638 and −498 functions as the core promoter essential for transcriptional activation of *hST8Sia I* gene

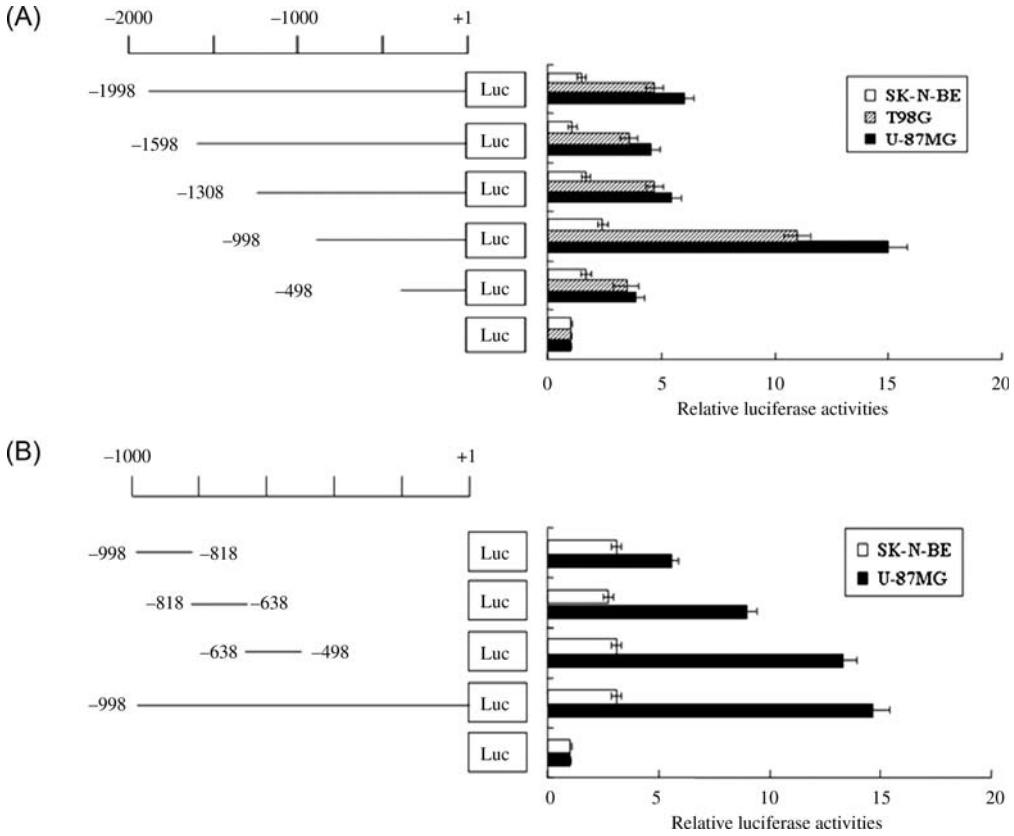

Fig. 4 Deletion analysis of *hST8Sia I* gene promoter in human glioblastoma cells The schematic diagrams represent DNA constructs (A) containing various lengths of the wild-type *hST8Sia I* promoter, or constructs (B) with same length deletions of the region from −998 to −498; the sequence is numbered with the first nucleotide of the transcription start site as +1. The pGL3-basic construct, which did not contain a promoter or an enhancer, was used as a negative control. Each construct was transfected into cells, with pRL-TK co-transfected as an internal control. Relative firefly luciferase activity was measured using the dual-luciferase reporter assay system, and all firefly luciferase activity was normalized to the *Renilla* luciferase activity derived from pRL-TK. The values represent the mean ± SD of three independent experiments with triplicate measurements.

in U-87MG cells. This also suggests that promoter elements located between nucleotide positions −638 and −498 are dominantly working for endogenous expression of the *hST8Sia I* gene in U-87MG cells.

Determination of the binding sites for transcriptional regulation of *hST8Sia I* gene in U-87MG cells

As shown in **Fig. 5(A)**, this region from −638 to −498 contains two putative binding sites, AREB6 and Elk-1. To determine whether these binding sites contribute to transcriptional regulation of *hST8Sia I* gene in U-87MG cell, three mutants (pGL3-638/-498mtAREB6, mtElk-1 and dmtAREB6/Elk-1) were prepared, which contains exactly the same construct as wild-type pGL3-638/-498, except that combined nucleotides within these binding sites had been changed. A series of substituted mutations of luciferase constructs were transfected into U-87MG and SK-N-BE(2)-C cells and luciferase assays were carried out. The activity of each construct was compared with those of pGL3-basic and wild-type (pGL3-638/-498) as negative and positive controls, respectively. In U-87MG cells, both pGL3-638/-498mtAREB6 and pGL3-638/-498mtElk-1 markedly reduced transcriptional activity to more than 3-fold of pGL3-638/-498wt. In addition, the activity of the double mutant (pGL3-638/-498dmtAREB6/Elk-1) was decreased to the level of the single mutant (pGL3-638/-498mtAREB6 and pGL3-638/-498mtElk-1) [**Fig. 5(B)**]. These results show that both AREB6 and Elk-1 sites are crucial for the expression of *hST8Sia I* gene in U-87MG cells. These results also suggest that AREB6 and Elk-1 collaborate each other as indispensable components for the expression of *hST8Sia I* gene in U-87MG cells.

Discussion

In the present study, we have demonstrated that the expression level of *hST8Sia I* gene is specifically high in

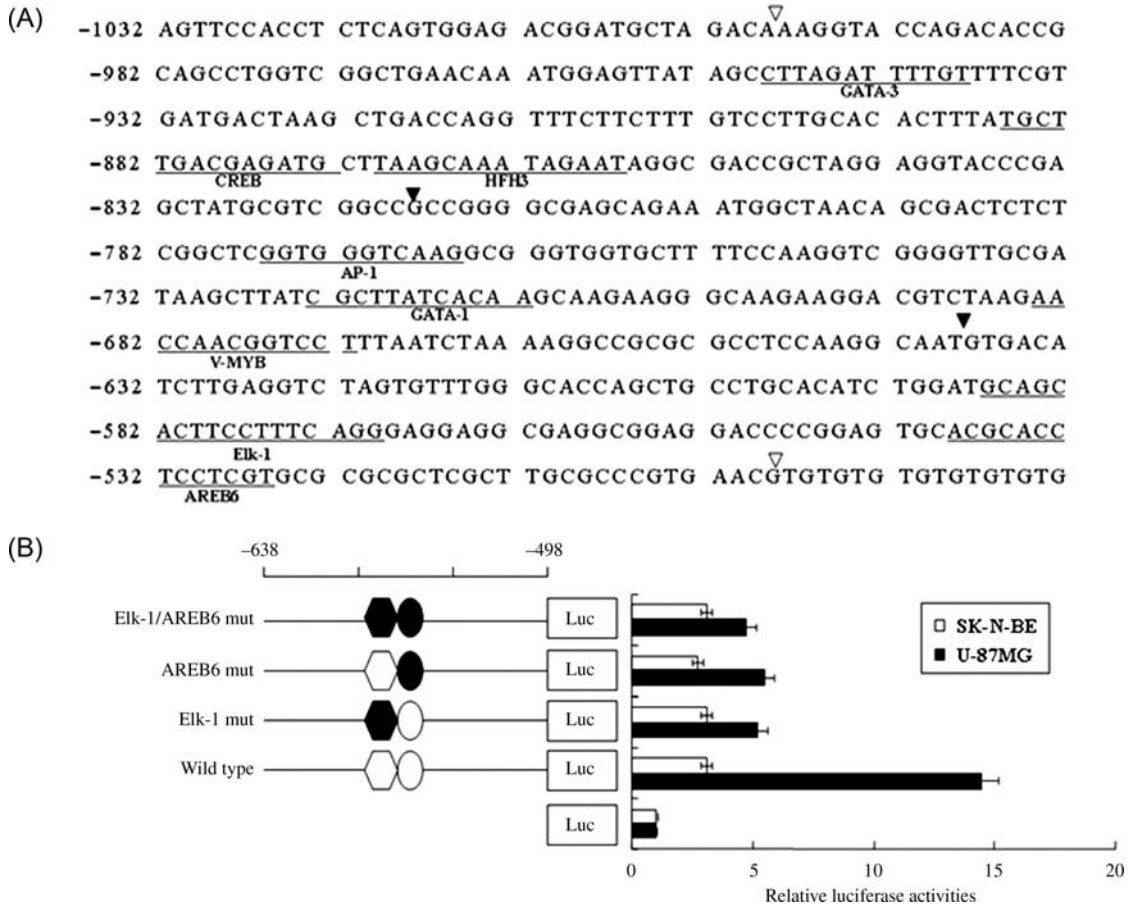

Fig. 5 Mutation promoter assay for the transcription factor-binding sites in promoter region of *hST8Sia I* gene (A) Nucleotide sequences of the promoter region from −1032 to −483 are shown. The numbering of the nucleotides begins with the A of ATG as +1. The opened arrowheads indicate the region from −998 to 498. The putative transcription factor binding sites are shown by underlines. For the deletion of promoter activity, the start point of each construction is indicated by closed arrowheads. (B) pGL3-Basic without any promoter and enhancer was used as a negative control. Each construct was co-transfected into U-87MG with pRL-TK co-transfected as an internal control. Relative luciferase activity was normalized with *Renilla* luciferase activity derived from pRL-TK. The values represent the mean ± SD for three independent experiments with triplicate measurements. The mutation mark of promoter construction is indicated by closed form or opened form (wild-type).

human glioblastoma cell lines. Our results obtained from RT-PCR and quantitative real-time PCR indicate that *hST8Sia I* mRNA is constitutively expressed in human glioblastoma cell lines T98G and U-87MG when compared with human neuroblastoma cell line SK-N-BE(2)-C and human Jurkat T-cells (**Fig. 1**). Previous studies have shown that malignant gliomas have high levels of GD3, and its expression correlates with the degree of malignancy [8,9]. As shown by immunofluorescent confocal microscopy, our present results also revealed that the levels of the ganglioside GD3 markedly increased in T98G and U-87MG cells when compared with SK-N-BE(2)-C cells. Recently, we reported the existence of a specific promoter region directing the transcription of *hST8Sia I* gene in human melanoma cells showing specifically high expression level of *hST8Sia I* gene [18].

To investigate promoter regions and functional elements important for transcriptional regulation of *hST8Sia I* gene expression in human glioblastoma cells, in this study, we undertook experiments to identify the glioblastoma-specific promoter sequence of the *hST8Sia I* gene and then to determine the transcription factors that regulate hST8Sia I gene expression in human glioblastoma cells. As the first step, we defined the 5′-terminal of the *hST8Sia I* mRNA obtained from U-87MG cells, because the *hST8Sia I* gene from human melanoma cell lines [18,19] as well as other human sialyltransferase genes [20] bears multiple potential transcription start sites. The 5′-RACE analysis, using mRNA isolated from U-87MG cells, showed a single product of about 0.7 kb [**Fig. 3(A)**]. The sequence analysis of this product also showed the same transcription start site as

one of those found in human melanoma SK-MEL-2 cells [18]. The 5′-terminal of the predominant 5′-RACE product was mapped at −648 bp from the translation initiation site. The analysis of the 2.0-kb nucleotide sequence of the 5′-flanking region revealed that unlike other human sialyltransferase genes, neither TATA and CAAT boxes nor a typical Sp1-binding site related to GC boxes was at the site appropriate to the transcription start site of the *hST8Sia I* gene in these cells, whereas it contained a number of putative regulatory *cis*-acting elements [18,19]. By functional analysis using transient transfection assays, we have demonstrated that the 5′-flanking region (pGL3-1998) of the *hST8Sia I* gene contained a functional promoter showing a high activity in human glioblastoma T98G and U-87MG cells and that the region between −998 and +1 functions as the major promoter essential for transcriptional activation of *hST8Sia I* gene in these cells, as revealed by the deletion mutant analysis. Interestingly, this region is different from those found in SK-MEL-2 cells [18] and Fas-induced Jurkat T cells [17], in which the region between −498 and +1 functions as the core promoter essential for transcriptional activation of *hST8Sia I* gene. In addition, further deletion mutant analysis showed that the region between −638 and −498 functions as the core promoter essential for transcriptional activation of hST8Sia I gene in U-87MG cells. In this region, there are two regulatory elements AREB6 (nt −539 to −526) and Elk-1 (nt −587 to −570).

It is important to characterize the two transcription factors in this region that might modulate the transcriptional activation of the *hST8Sia I* gene in U-87MG cells, because they are known to be involved in development [21], apoptosis [22], and cell proliferation of glioblastoma [23]. Our results by site-directed mutagenesis clearly showed that mutations of AREB6 and Elk-1 binding sites resulted in a significant decrease of promoter activity to *hST8Sia I* gene [**Fig. 5(B)**]. These results indicated that both AREB6 and Elk-1 elements were crucial for the transcription of *hST8Sia I* gene in U-87MG cells, unlike in SK-MEL-2 [18] and Fas-induced Jurkat T cells [17] showing that NF-κB element at nucleotide positions −83 to −74 is the most functional for the transcription of *hST8Sia I* gene.

AREB6 is a zinc finger-homeodomain transcription factor that regulates the Na,K-ATPase α1 subunit gene (*Atp1a1*) positively or negatively depending on cell types [21,24]. AREB6 plays important roles in the expression of tissue-specific genes and in various developmental processes [21]. However, its physiological function was not studied extensively, when compared with other transcription factors.

Elk-1 belongs to the ETS-domain transcription factor family and the ternary complex factor (TCF) subfamily [25]. Elk-1 is known to be implicated in neuronal differentiation, cellular proliferation and apoptotic cell death [22,25]. It is also established that the TCFs are direct targets of the mitogen-activated protein kinases and Elk-1 is regulated by extracellular signal-regulated kinases (ERKs) [25]. Moreover, a recent study has shown that proliferation of glioblastoma cells was induced by protein kinase C-η isoform through ERK/Elk-1 pathway [23]. Therefore, it is possible that the high constitutive activation of Elk-1 in U-87MG cells triggers the high constitutive expression of hST8Sia I mRNA in these cells.

Although the precise mechanisms involved in the constitutive activation of AREB6 and Elk-1 leading to a transcriptional up-regulation of *hST8Sia I* gene in human glioblastoma cells are unknown, in the present study, we have shown for the first time that both AREB6 and Elk-1 are crucial for the *hST8Sia I* gene expression necessary for ganglioside GD3 synthesis in U-87MG cells. Further experiments, including electrophoretic mobility shift assay are needed to confirm involvement of these elements in the human glioblastoma-specific expression of the *hST8Sia I* gene.

Funding

This study was supported by a grant form the Research Funds from Dong-A University.

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