Characterization of the human sialidase Neu4 gene promoter

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Abstract: There are 4 different sialidases that have been described in humans: lysosomal (Neu1), cytoplasmic (Neu2), plasma membrane (Neu3), and lysosomal/mitochondrial (Neu4). Previously, we have shown that Neu4 has a broad substrate specificity and is active against glyco-conjugates, including G_{M2} ganglioside, at the acidic pH of 3.2. An overexpression of Neu4 in transfected neuroglia cells from a Tay–Sachs patient shows a clearance of accumulated G_{M2}, indicating the biological importance of Neu4. In this paper, we aimed to characterize a minimal promoter region of the human Neu4 gene in order to understand the molecular mechanism regulating its expression. We cloned 7 different DNA fragments from the human Neu4 promoter region into luciferase expression vectors for a reporter assay and also performed an electrophoretic mobility shift assay to demonstrate the binding of transcription factors. We demonstrated that –187 bp upstream of the Neu4 gene is a minimal promoter region for controlling transcription from the human Neu4 gene. The electrophoretic mobility shift assay showed that the minimal promoter region recruits a c-myc transcription factor, which might be responsible for regulation of Neu4 gene transcription. The data we obtained might be useful to discover small molecules, which control selective high expression of the human Neu4 gene, resulting in the normal morphological phenotype in the lysosomes of Tay–Sachs patients.

Key words: Sialidase, Neu4, promoter, protein-DNA interaction, transcription factors, c-myc

1. Introduction

Sialidases (EC 3.2.1.18), also known as neuraminidases, are glycohydrolytic enzymes responsible for removing sialic acid residue from sialylated glycoproteins, glycolipids, and oligosaccharides (Saito and Yu, 1995). Sialidases have a wide distribution in nature among viruses, bacteria, protozoa, and vertebrates (Svennerholm, 1963; Saito and Yu, 1995). So far, 4 types of sialidases have been identified in humans and characterized according to their subcellular distribution, substrate specificity, and stability. These sialidases are lysosomal Neu1, cytosolic Neu2, plasma membrane Neu3, and lysosomal/mitochondrial membrane Neu4 (Seyrantepe et al., 2004).

The sialidase Neu4 enzyme is expressed by the gene NEU4 (NCBI Gene ID: 129807), which maps in the telomeric region of the long arm of the human chromosome 2 (2q37). The NEU4 gene was discovered as a result of a sequence database search that revealed homology to the human cytosolic sialidase NEU2 gene (Monti et al., 2004). The sialidase Neu4 enzyme has the highest expression level in the liver and it is also ubiquitously expressed in all central nervous system districts, the colon, the small intestine, the kidney, the heart, skeletal muscle, and the placenta. Targeting of the sialidase Neu4 enzyme to lysosomes by the mannose 6-phospate receptor has been shown (Seyrantepe et al., 2004). It has also been shown that the sialidase Neu4 enzyme localizes in the lysosomal lumen as a soluble hydrolase. The sialidase Neu4 enzyme has 2 major isoforms: a short form containing 484 amino acids and a long form containing 496 amino acids. It has been suggested that the long form localizes in mitochondria and the short form is associated with the endoplasmic reticulum (Bigi et al., 2010). Seyrantepe et al. (2004) first biochemically characterized the Neu4 enzyme, which has an almost equal broad substrate specificity against glycoproteins (mucin), oligosaccharides (sialyllactose) and sialylated glycolipids (mixed bovine gangliosides), and synthetic substrates 2'-(4-methylumbelliferyl)-alpha-D-N-acetylneuraminic acid (4-MU-NANA or 4MU-NeuAc) at an acidic pH of 3.2 (Monti et al., 2004; Seyrantepe et al., 2004). Overexpression of the sialidase Neu4 enzyme in sialidase Neu1-deficient sialidosis fibroblasts has been associated with the clearance of accumulated substrates and normal morphological phenotype of the lysosomal compartment. In addition, the complete elimination of storage materials in sialidosis and of galactosialidosis

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cells was achieved by only 3–5% of Neu4-expressing cells, indicating the therapeutic potential of the sialidase Neu4 enzyme in sialidosis and galactosialidosis for enzyme replacement therapy (Seyrantepe et al., 2004). Sialidase Neu4-deficient (Neu4$^{-/-}$) mice showed vacuolization and lysosomal storage in lung and spleen cells. Neu4$^{-/-}$ mice also have increased levels of G$_{D1a}$ ganglioside and decreased levels of G$_{M1}$ ganglioside in the brain, which supports the evidence for sialidase Neu4 enzyme desialylation activity against brain gangliosides (Seyrantepe et al., 2008). It has been shown that Neu4 is downregulated in human colon cancer cells and that the overexpression of Neu4 in cultured cells accelerates apoptosis and decreases invasiveness and motility (Miyagi, 2008).

Tay–Sachs disease, which is due to a deficiency of the β-hexosaminidase A enzyme, causes an accumulation of G$_{M2}$ ganglioside in lysosomes resulting in neurodegeneration (Kolodny, 1966). Mouse models of Tay–Sachs disease have been generated by targeted disruption of the HexA gene. Analysis of these knockout mice revealed that although G$_{M2}$ ganglioside accumulated in the brain, the mice did not present any kind of human Tay–Sachs phenotype-like behavioral or motor abnormalities for at least 1 year. It has been suggested that sialidase(s) in HexA-deficient (HexA$^{-/-}$) mice can convert G$_{M2}$ to G$_{A2}$ ganglioside, which is an asialo-G$_{M2}$ form, and then to lactocyl ceramide by the HexB isoenzyme (Cohen-Tannoudji et al., 1995; Sango et al. 1995; Phaneuf et al., 1996). This metabolic bypass prevents mice from presenting an early phenotype of Tay–Sachs disease. However, we previously showed that mice deficient in both HexA and Neu4 exhibit epileptic crises, degenerating neurons, motor impairment-like tremors, weakness, and spasticity. In addition, elevated G$_{M2}$ ganglioside accumulation has been observed in comparison to single knockout HexA$^{-/-}$ or Neu4$^{-/-}$ mice (Seyrantepe et al., 2010). The data revealed that due to a sialidase Neu4 enzyme deficiency HexA$^{-/-}$ mice show a severe phenotype supporting the modifier role of the sialidase Neu4 enzyme in metabolic bypass in this Tay–Sachs disease mouse model. All these data indicate that the sialidase Neu4 enzyme might be a potential therapeutic modifier by a selective pharmacologic induction through its upregulation with small molecules (Seyrantepe et al., 2008, 2010).

In this work, we aimed to characterize the promoter region of the human sialidase NEU4 gene, which has not yet been reported. We also showed specific interactions of transcription factor c-myc in the promoter region, which has the potential to be a regulator of Neu 4 gene expression.

2. Materials and methods

2.1. Bioinformatics
The –3000 bp upstream region from the human NEU4 gene start codon (ATG) was considered to be a region containing the regulatory elements for the Neu4 gene. The sequence of –3000 bp was analyzed using the Transcription Element Search System (TESS) tool for the mapping of transcription binding sites.

2.2. Plasmid construction
Seven pairs of primers (F1:5'-GGTACCCTCCTGGGTGCCCATCTG-3', F2:5'-GGTAC CTCTCTGGAGCAGCAGACC-3', F3:5'-GGTACCGTGAATGGGACTGGCAGGAG-3', F4:5'-GGTACCGGGGGAAGCTTTCCTTAACC-3', F5:5'-GGTACCACACCCTGGCCAG ACAGC-3, F6:5'-GGTACCCCTGTCCCTGAGCGGAAC-3', F7:5'-GGTACCCTCCGTGT CAGTGTGCATTC- 3', Reverse primer: 5'- GCTAGCGCTGCAGAGCTCATCATGG- 3') were designed to obtain overlapping DNA fragments 187 bp, 358 bp, 591 bp, 1198 bp, 1592 bp, 2364 bp, and 3000 bp in length. Human genomic DNA was amplified by PCR with i-Taq DNA Polymerase (Intron Biotechnology) and cloned into the pCR 2.1 TOPO vector using the TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's instructions. Seven different fragments were subcloned into the pGL 4.12 (Firefly) plasmid with NheI and KpnI restriction sites from the pCR 2.1 TOPO vector using T4 DNA Ligase (Invitrogen).

2.3. Cell culture, transient transfection, and luciferase assay
HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS (heat-inactivated), 1% penicillin/streptomycin, and 2 mM L-glutamine in a humidified incubator at 37 °C under 5% CO$_2$. HeLa cells were inoculated in 96-well plates at a density of 1×10^4 cells/well to provide 90% confluency prior to transfection before 24 h. Turbofect in vitro transfection reagent (Fermentas) was used according to the manufacturer's instructions for all of the transfections. All reporter assays were performed 3 times and included cells without transfection as blanks. The cells were also co-transfected with both a promoter-less empty pGL 4.12 vector and a pGL 4.74 vector as a negative control. The luciferase assay was optimized using different amounts of DNA molecules and different incubation times. According to optimization assays, the cells were co-transfected with 210 ng of pGL 4.12 (Promega), which contained 7 different DNA fragments as a promoter construct and 7 ng of pGL 4.74 (Promega), which expresses renilla luciferase as an internal control for transfection efficiency using Turbofect

transfection reagent (Fermentas) without serum. After transfection, the cells were incubated at 37 °C under 5% CO_2 for 4 h, then replaced with fresh medium, and incubated for 24 h for the reporter assay. The cells were washed with PBS (phosphate buffer saline) and lysed with 20 μL of 1X Passive Lysis Buffer (Promega) by shaking for 15 min on an orbital shaker. After the cells were lysed, luciferase activities were measured with a plate-reading luminometer (Thermo, VarioSkan) using a Dual Luciferase Reporter Assay System kit (Promega) according to the manufacturer's instructions by injecting 100 μL of both Dual Luciferase Reporter Assay System reagents into 20 μL of cell lysate. Luciferase activities were calculated as a ratio of pGL 4.12 to pGL 4.74, revealing a promoter activity relative to pGL 4.12 basic.

2.4. Nuclear extract preparation and electrophoretic mobility shift assay

Nuclear proteins were isolated from HeLa cells using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). Concentrations of the isolated proteins were determined using a Pierce BCA Protein Assay Kit (Thermo Scientific). Fragments were amplified from the 5' upstream region of the NEU4 gene to produce double stranded probes with biotin. A Biotin 3' End DNA Labeling Kit (Thermo Scientific) was used to biotinylate 5 pmol of the 3' ends of the F1 fragment (187 bp), F2 fragment (358 bp), and F3 fragment (591 bp). An electrophoretic mobility shift assay (EMSA) was performed using a LightShift Chemiluminescent EMSA Kit (Thermo Scientific). Binding reactions included 1X binding buffer supplied with the kit, 5 μg of nuclear extract, 5 pmol of biotin-labeled DNA fragments, and 50 ng/μL of salmon sperm, followed by incubation at room temperature for 20 min. For competition assays, binding reactions included 1X binding buffer supplied with the kit, 5 μg of nuclear extract, and a 50-fold excess of unlabeled DNA fragments (F1, F2, F3 fragments for relative reactions) as competitor. These were pre-incubated for 5 min at room temperature, then 5 pmol of biotinylated DNA fragments was added and incubated for an additional 20 min at room temperature to demonstrate the specificity of DNA–protein interactions. For the supershift assay, 2.5 μg of an antibody (Sigma) directed against the transcription factor c-myc was incubated with nuclear extract on ice for 1 h, then added into the binding reaction prior to the addition of the biotin-labeled DNA. Protein–DNA complexes were separated on 3.5% native polyacrylamide gel electrophoresis in 0.5X Tris-Borate-EDTA buffer for 1 h and then transferred to a positively charged nylon membrane (HyBond-N⁺) in 0.5X Tris-Borate-EDTA buffer for 1 h. Protein–DNA complexes were cross-linked to the membrane at 120 mJ/cm² of UV light and detected using the Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific).

3. Results

Sequence analysis of the –3000 bp upstream region of the human Neu4 gene using the TESS tool revealed that the Neu4 promoter harbored several transcription factor binding sites (C/EBP, CREB, AP-1, c-myc, c-myb, SP-1, cEtc-2, YY1, and MyoD) and the absence of a TATA box. In addition, the analysis of GC boxes using Molecular Biology Core Facilities (MBCF) Oligo Calculator revealed that the region –3000 bp upstream of human Neu4 has 61% GC content (Figures 1A and 1B).

The promoter activity of the human Neu4 gene was studied by transient transfection in HeLa cells. In order to characterize the upstream regulators, we used a dual luciferase reporter system because of its experimental accuracy and high sensitivity (as low as femtograms of luciferase). Amplified DNA fragments were subcloned into a pGL 4.12 vector. We confirmed that there was no DNA polymerase error in the insert by sequencing (data not shown). We also optimized the assay parameters and found that the ratio of 30:1 (pGL 4.12:pGL 4.74) was optimal for the Dual Luciferase Reporter System. All reporter assays were repeated 3 times. Luciferase activities are shown as a relative fold activity change to promoter-less pGL 4.12 vector. The region located on –187 bp (F1) showed minimal promoter activity and high luciferase expression, indicating the importance of potential regulatory elements such as c-myc (Figure 1C).

The reporters containing the –187 bp (F1), –358 bp (F2), –591 bp (F3), and –1187 bp (F4) fragments of sequence all exhibited similar, high levels of luciferase expression. Therefore, in order to examine the association of transcription factors with putative binding sites in the promoter region, we used only the –187 bp (F1), –358 bp (F2), and –591 bp (F3) fragments as probes for EMSA. We showed the presence of transcription factor binding site(s) by shift formation, as illustrated in Figure 2. Specific shift formation was only detected for the F1 fragment (A) and to some extent for F2 (B). This is due to the fact that we had difficulty obtaining well resolved results for F3 (C) in the competition assays, most likely because the competitor we used successfully degraded the F3 shift and reduced the intensity. Therefore, specific transcriptional factor bindings to the F3 fragment, when combined with the competitor, were disrupted and the F3-protein complex degraded.

To determine the interaction of transcription factor c-myc with its binding site, which is located –114 bp from the start codon (ATG) and has the sequence of CACCGT, we performed a supershift assay using the anti c-myc antibody. As shown in Figure 3, we found that c-myc specifically bound to the –187 bp upstream region.
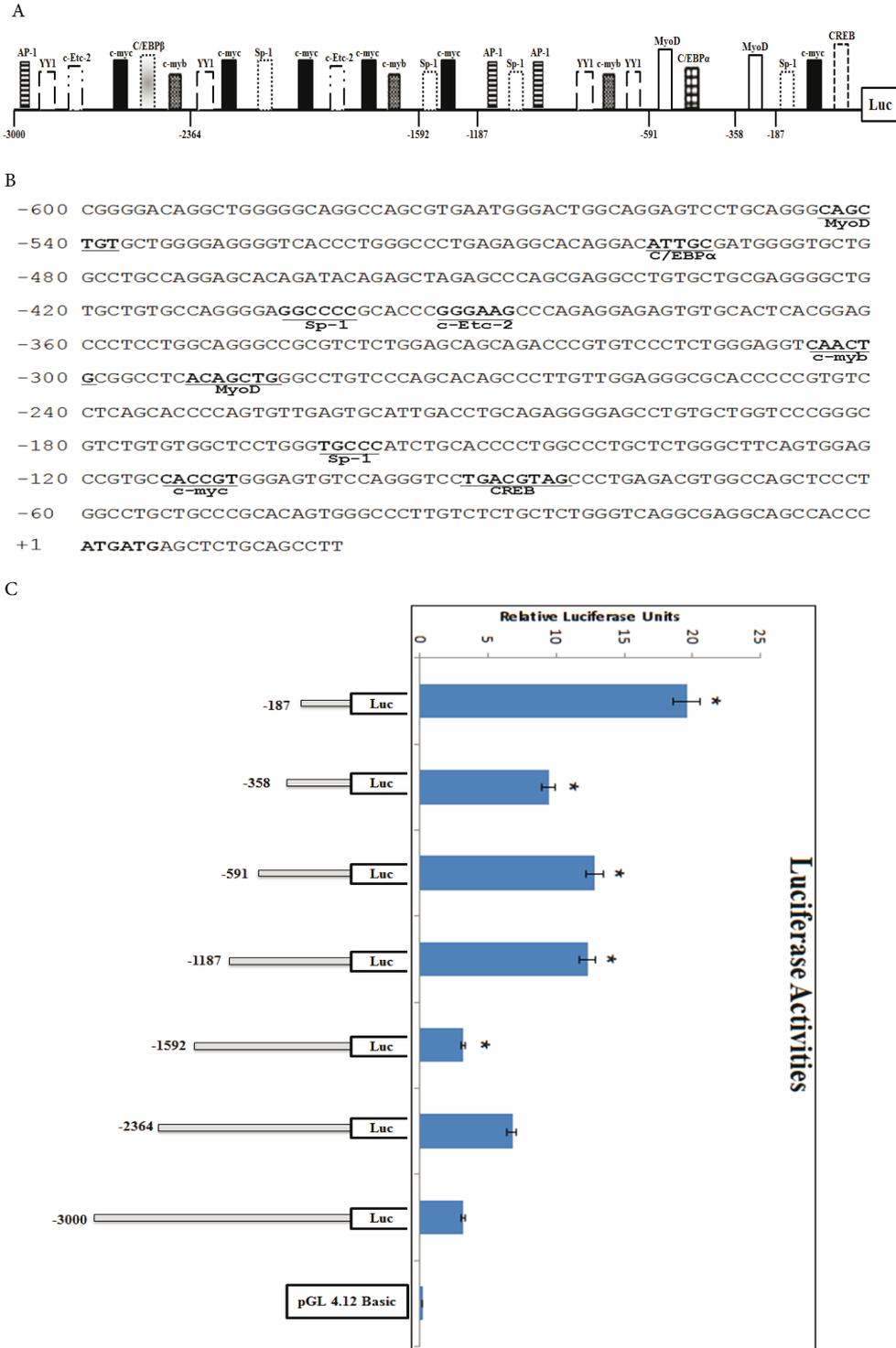
A

B

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-600  CGGGGACAGGCTGGGGGCAGGCCAGCGTGAATGGGACTGGCAGGAGTCCTGCAGGGCAGC
                                                               MyoD
-540  TGTGCTGGGGAGGGGTCACCCTGGGCCCTGAGAGGCACAGGACATTGCGATGGGGTGCTG
                                                        C/EBPα
-480  GCCTGCCAGGAGCACAGATACAGAGCTAGAGCCCAGCGAGGCCTGTGCTGCGAGGGGCTG
-420  TGCTGTGCCAGGGGAGGCCCCGCACCCGGGAAGCCCAGAGGAGAGTGTGCACTCACGGAG
                          Sp-1        c-Etc-2
-360  CCCTCCTGGCAGGGCCGCGTCTCTGGAGCAGCAGACCCGTGTCCCTCTGGGAGGTCAACT
                                                               c-myb
-300  GCGGCCTCACAGCTGGGCCTGTCCCAGCACAGCCCTTGTTGGAGGGCGCACCCCCGTGTC
           MyoD
-240  CTCAGCACCCCAGTGTTGAGTGCATTGACCTGCAGAGGGGAGCCTGTGCTGGTCCCGGGC
-180  GTCTGTGTGGCTCCTGGGTGCCCATCTGCACCCCTGGCCCTGCTCTGGGCTTCAGTGGAG
                          Sp-1
-120  CCGTGCCCACCGTGGGAGTGTCCAGGGTCCTGACGTAGCCCTGAGACGTGGCCAGCTCCCT
            c-myc                  CREB
-60   GGCCTGCTGCCCGCACAGTGGGCCCTTGTCTCTGCTCTGGGTCAGGCGAGGCAGCCACCC
+1    ATGATGAGCTCTGCAGCCTT
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C

Figure 1. A DNA sequence analysis of the Neu4 promoter showing high score transcription binding site predictions (A). Sequence analyses were performed using TESS algorithms. Potential transcription factor binding sites are underlined (B). Luciferase assays of deleted fragments were performed in triplicate (C). Each value is expressed as mean ± standard deviations. Fisher's least squared significant difference test was performed for post-hoc analysis following ANOVA. Statistical significance was determined as $*P < 0.05$ compared to the control.
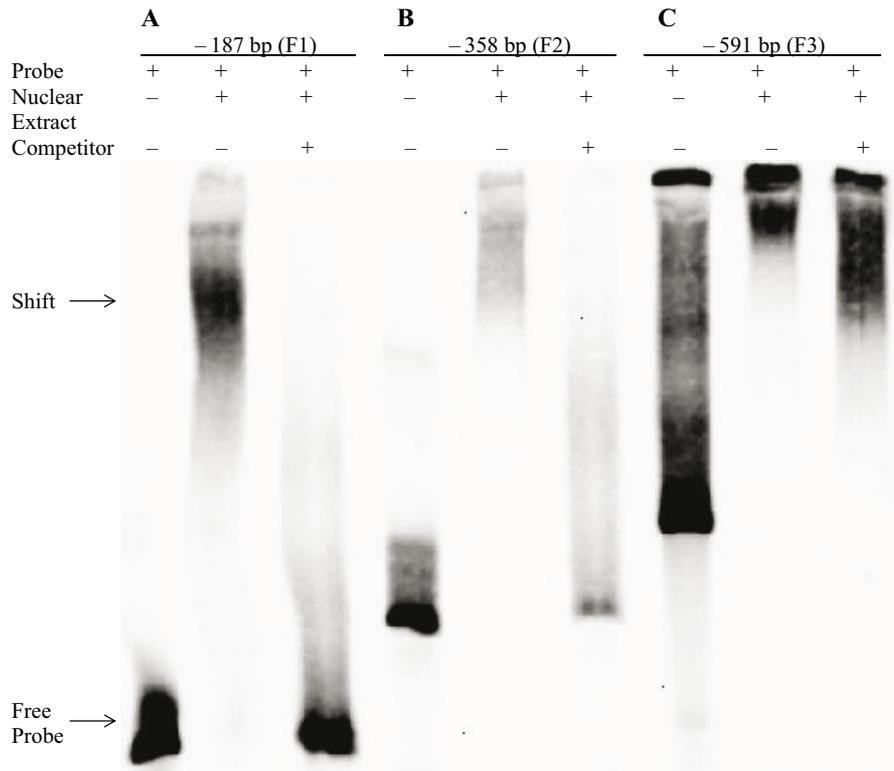
Figure 2. An image showing the EMSA results of the (A) –187 bp upstream region (F1), (B) –358 bp upstream region (F2) and (C) –591 bp upstream region (F3). Lane 1: free biotin labeled probes without nuclear extract; lane 2: labeled probes with 5 μg of nuclear extract; lane 3: labeled probes with nuclear extract and 50-fold excess of unlabeled probe for competition assay.

4. Discussion

Sialidases (neuraminidases) are glycohydrolytic enzymes that remove sialic acid residues from glycoconjugates (Saito and Yu, 1995). They show a wide distribution in nature, from viruses to vertebrates. In humans, 4 types of sialidases have been identified and cloned so far. It has been shown that lysosomal/mitochondrial sialidase 4 (Neu4) has activity against sialylated glycoproteins, oligosaccharides, and glycolipids in vitro. In Neu4 transfected Tay–Sachs neuroglia cells, clearance of accumulated G_{M2} ganglioside has been shown (Seyrantepe et al., 2004). Neu4 deficient mice also show an increased G_{D1a} and a decrease in G_{M1} ganglioside levels in the brain, indicating the activity of Neu4 against gangliosides in vivo (Seyrantepe et al., 2008).

In this study, our aim was to characterize the 5' upstream regulatory region of the human Neu4 sialidase gene. Although the biochemical properties of the human sialidase Neu4 enzyme have previously been reported, the Neu4 promoter region has not been characterized yet. Our data provided an important clue about the molecular mechanism regulating human Neu4 gene expression. We focused on the –3000 bp upstream region of the human Neu4 gene as a candidate promoter region. Bioinformatic analysis using TESS tool revealed several putative transcription factor binding sites such as c-myc, CREB, SP-1, YY1, and AP-1 in the TATA-less promoter. The data obtained using the Dual Luciferase Reporter System showed, by demonstrating the highest luminescence among all the bp fragment regions, that the –187 bp upstream region (F1 fragment) has minimal transcriptional regulatory activity for the human Neu4 gene. We found that the fold activity change of luminescence gradually decreased between the –187 bp and –1187 bp region. On the other hand, the –358 bp upstream region showed a significant decrease in fold activities due to potential elements probably responsible for the downregulation of Neu4 gene. In addition, significantly decreased fold activities of luminescence were detected in the –1592 bp, –2364 bp, and –3000 bp upstream regions due to the effect of potential silencer elements. The –187 bp region (F1 fragment) has dominant promoter activity by showing 6 times higher fold activity than the –1592 bp region (F5 fragment). EMSA revealed that the –187 bp, –358 bp, and –591 bp upstream regions recruited some transcription factors as seen in "shift"
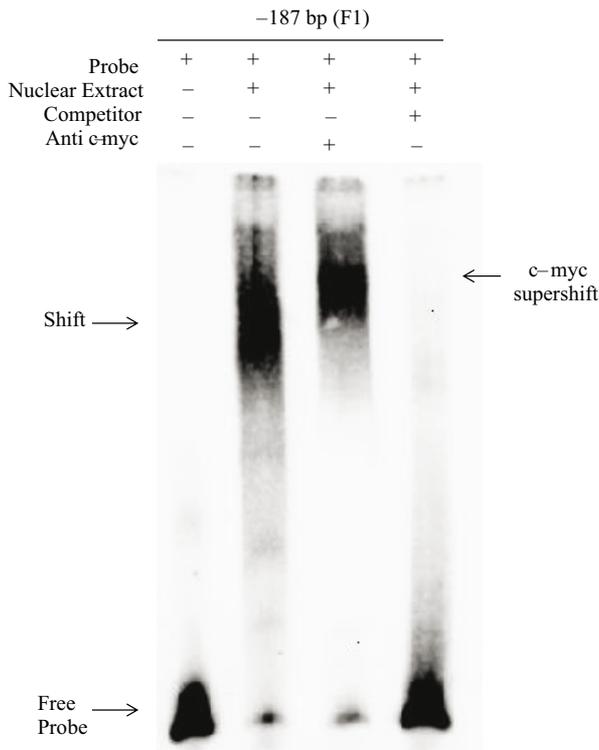
Figure 3. An image showing the result of the –187 bp upstream region (F1) with c-myc supershift. Lane 1: free biotin labeled probes without nuclear extract; lane 2: labeled probes with 5 µg of nuclear extract; lane 3: labeled probes with nuclear extract and 2.5 µg of antibody (Sigma) against transcription factor c-myc for supershift assay; lane 4: labeled probes with nuclear extract and 50-fold excess of unlabeled probe for competition assay.

formation with the incubation of biotinlated DNA and nuclear proteins isolated from HeLa cells. The addition of the unlabeled 187 bp, 358 bp, and 591 bp fragments as competitors into relative binding reactions proved the specificity of the binding of transcription factors to the candidate promoter region. EMSA also confirmed further supporting evidence for the regulatory role of the –187 bp upstream region (F1 fragment), as we determined the

formation of a "specific shift" at the protein level, besides its highest luminescence fold activity. In particular, we showed the binding of transcription factor c-myc to our minimal promoter region by supershift assay. The highest luminescence obtained from the –187 bp upstream region (F1 fragment) of the human Neu4 gene may be a result of the predicted transcription factor binding sites for c-myc, which are also responsible for transcriptional upregulation of many genes involved in cellular events such as cell proliferation and differentiation.

In the future, the data we presented in this paper can be used to discover small molecules that may regulate human Neu4 gene expression. Selective high expression or silencing of the Neu4 gene might be achieved by using drugs or small molecules. High expression of the Neu4 gene and increased levels of sialidase Neu4 enzyme activity in tissues can clear accumulated G_{M2} ganglioside in the lysosomes of Tay–Sachs patients caused by β-hexosaminidase A deficiency.

It has been shown that Neu4 is downregulated in human colon cancer cells and that the overexpression of Neu4 in cultured cells accelerates apoptosis and decreases invasiveness and motility. Research directed at studying the upregulation of human Neu4 gene expression by selective binding of c-myc to the promoter region might be also important to treat human colon cancer. Finally, the importance of up and/or downregulation of Neu4 gene expression in the degradation of biomolecules, cellular communication, cell growth and differentiation, and cell death would be clarified.

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