MOLECULAR ANALYSIS OF MAMMALIAN NEU4 SIALIDASE GENE PROMOTER

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ABSTRACT

MOLECULAR ANALYSIS OF MAMMALIAN NEU4 SIALIDASE GENE PROMOTER

There are four different mammalian sialidases that have been described; lysosomal (Neu1), cytoplasmic (Neu2), plasma membrane (Neu3), lysosomal/mitochondrial (Neu4). The activity of sialidase Neu4 enzyme against sialic acid containing ganglioside G_{M2} has been demonstrated. Biological role of sialidase Neu4 enzyme has been shown by the transfection of neuroglia cells from a Tay-Sachs patient with a Neu4-expressing plasmid showed clearance of accumulated ganglioside G_{M2}. It has been also shown that sialidase Neu4 enzyme is responsible for degradation reactions of another ganglioside such as G_{D1a} in brains of $Neu4^{-/-}$ mice. Aim of our study is to identify minimal promoter region of human Neu4 gene and demonstrate binding of transcription factors to this region.

In our study, we used bioinformatic approaches to predict the sequence motifs where several specific transcription factors bind using TESS (Transcription Element Seach System) tool. We amplified seven different DNA fragments from human Neu4 promoter region, cloned into luciferase expression vector and performed reporter assay. We also performed electrophoretic mobility shift assay to demonstrate binding of transcription factors to candidate promoter region.

We demonstrated that 187 bp upstream of Neu4 gene is minimal promoter region to control transcription from Neu4 gene. Electrophoretic mobility shift assay showed that 187 bp upstream region recruits several transcription factors.

Our results demonstrated the minimal promoter region revealing several putative transcription factors such as Sp-1 and c-myc which might be responsible mainly for regulation of Neu4 gene transcription. The data we obtained might be useful to discover small molecules which can control Neu4 gene expression. High expression of Neu4 gene might be controlled using drugs or small molecules and the accumulated G_{M2} ganglioside in lysosomes of Tay-Sachs patients can be reduced.

ÖZET

MEMELİ NEU4 SİALİDAZ GEN PROMOTÖRÜNÜN MOLEKÜLER ANALİZİ

Memelilerde tanımlanmış dört farklı sialidaz enzimi bulunmaktadır. Bunlar; lizozomal (Neu1), sitoplazmik (Neu2), plazma membran (Neu3), lizozomal/mitokondrial (Neu4) sialidaz olarak sınıflandırılmıştır. Sialidaz Neu4 enziminin, sialik asit içeren G_{M2} gangliozidine karşı olan aktivitesi gösterilmiştir. Sialidaz Neu4 enziminin biyolojik rolü Tay-Sachs hastasından alınan nöroglia hücrelerinin Neu4 ifade eden plazmid ile transfekte edilmesi sonucunda biriken G_{M2} gangliozidlerin yıkıma uğraması ile gösterilmiştir. Bunun yanı sıra $Neu4^{-/-}$ fare beyinlerinde sialidaz Neu4 enziminin G_{D1a} gangliozidinin yıkımından sorumlu olduguda gösterilmiştir.

Çalışmamızda sialidaz Neu4 geninin minimal promotör bölgesinin moleküler analizi ve transkripsiyon faktörlerinin bu bölgeye bağlanması araştırılmıştır.

Araştırmamızda, TESS (Transcription Element Seach System) programı kullanılarak bazı spesifik transkripsiyon faktörünün bağlandığı sekans motifleri biyoinformatik yöntemlerle tahmin edilmiştir. Yedi farklı DNA fragmenti insan Neu4 promotör bölgesinden amplifiye edilip lusiferaz ekspresyon vektörüne klonlanarak lüminometrik ölçüm yapılmıştır. Transkripsiyon faktörlerinin muhtemel promotör bölgesine bağlanmasını göstermek için DNA:protein ilişkisini gösteren EMSA (electrophoretic mobility shift assay) deneyi gerçekleştirilmiştir.

Neu4 geninin 187 baz çifti önünde yer alan bölgenin minimal promotör bölgesi olduğu gösterilmiştir. Transkripsiyon faktörlerinin 187 baz çiftlik bölgeye bağlandıkları kanıtlanmıştır.

Minimal promotör bölge aydınlatılıp Neu4 geninin transkripsiyonunun regülasyonundan sorumlu olabilecek c-myc ve Sp-1 gibi transkripsiyon faktörleri belirlenmiştir. Elde edilen veriler gelecekte Neu4 geninin kontrolünü sağlayan küçük moleküllerin sentezlenmesinde rol oynayabilecektir. Neu4 geninin küçük moleküllerle veya ilaçlar ile ifadesinin artırılması Tay-Sachs hastalarının lizozomlarında biriken G_{M2} gangliozidinin yıkımı sağlanabilecektir.

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CHAPTER 1

INTRODUCTION

1.1. Neuraminidases

Neuraminidases (EC 3.2.1.18), also known as sialidases, are glycohydrolytic enzymes that are responsible for removing sialic acid residues from sialylated glycoproteins, glycolipids and oligosaccharides (Magesh, Miyagi et al. 2006; Miyagi 2008; Seyrantepe et al. 2004). Neuraminidases cause a conformational change in glycoconjugate structure by removing the sialic acid groups and this conformational change induce metabolic processes via recognition of these molecules (Miyagi 2008). It has been shown that neuraminidases are expressed in all cell types and tissues and involved in metabolic processes like cell proliferation, adhesion, differentiation, catabolism of glycolipids and glycoproteins, membrane fusion and fluidity (Saito and Yu, 1995; Bonten 2000). Neuraminidases have a wide distribution in nature among viruses, bacteria, protozoa and vertebrates (Saito and Yu 1995). In humans, four types of neuraminidases have been identified and characterized according to their subcellular distribution, substrate specificity and stability (Magesh and Miyagi et al. 2006; Bonten 2000).These neuraminidases are lysosomal Neu1 (Bonten 1996), cytosolic Neu2 (Monti 1999), plasma membrane Neu3 (Monti et al. 2000) and lysosomal or mitochondrial membrane Neu4 (Monti et al. 2004). Although neuraminidases differ in substrate specificity, all human neuraminidases contain conserved active sites, the F/YRIV/P motif, in the N-terminal part and the Asp boxes (Roggentin et al. 1993).

1.1.1. Neuraminidase 1

Neu1 is expressed from the gene Neu1 which is located on human chromosome 6p21.3 (Bonten et al. 1996) and it has 6 exons spaning approximately 3.5 kb of genomic DNA (Milner et al. 1997). Vesicular transport system which is responsible for trafficking of molecules between different membrane-enclosed compartments in a cell

targets Neu1 to the endosomal-lysosomal system to be an integral membrane protein (Lukong et al. 2001). Subcellular locations of Neu1 can be listed as lysosomal membrane, peripheral membrane, lysosome lumen, cell membrane (Lukong and Seyrantepe et al. 2001). It is found in all vertebrate cells and tissue types like brain, kidney, liver, testis (Saito et al. 1995). It is mostly expressed in pancreas but weakly expressed in brain (Uniprot, 2011). Neu 1 is associated with β-galactosidase and Cathepsin A in lysosomes and loss of this multienzyme complex causes reduction in Neu1 activity (Pshezhetsky et al. 1997). Neu1 is responsible for catalyzing the removal of sialic acid from glycoproteins and glycolipids hydrolysing of alpha-(2→3), alpha-(2→6), alpha-(2→8) glycosidic linkages of terminal sialic acid residues. It has a narrow substrate specificity against oligosaccharides, glycopeptides and artificial substrates like 4MU-Neu5Ac (4-methylumbelliferyl-N-acetylneuraminic acid) (Pshezhetsky et al. 2001). Another important role of Neu1 is cellular immune response during monocyte differentiation by relocalizing from lysosomes to cell surface (Liang and Seyrantepe et al. 2006). Defects in Neu1 gene causes a neurodegenerative disorder called sialidosis which is an autosomal recessive lysosomal storage disease. Neu1 deficiency cause accumulation of sialylated oligosaccharides in the lysosome (Thomas et al. 2001). Sialidosis has two types; Sialidosis type I (the mild and late onset form) which shows gait abnormalities, progressive impaired vision, bilateral macular cherry-red spots and myoclonus syndrome (Durand et al. 1977; Rapin et al. 1978; O'Brien 1979) and Sialidosis type II (severe, early-onset form) which shows dysostosis multiplex, short stature, developmental delay, mental retardation and hepatosplenomegaly (Kelly and Graetz 1977; Winter et al. 1980). The confirmation of the diagnosis is performed by screening of the urine for sialyloligosaccharides (Uniprot, 2011) and patients can also be diagnosed biochemically by measuring lysosomal enzyme activities in cultured skin fibroblasts or amniocytes (Suzuki 1987).

1.1.2. Neuraminidase 2

Neu2 is expressed from the gene Neu2 which localizes on human chromosome 2q37. It is located in cytoplasm and mostly expressed in skeletal muscle, fetal liver and embryonic carcinoma cell line NT2D1 (Uniprot, 2011). Neu2 hydrolyzes alpha-(2→3), alpha-(2→6), alpha-(2→8) glycosidic linkages of terminal sialic acid residues in

oligosaccharides, glycoproteins, glycolipids at approximatly neutral pH in contrast to lysosomal Neu1 sialidase (Miyagi et al. 1985; Tringali et al. 2004). Role of Neu2 in mammals is suggested in myotube formation (Sato 1996). The mechanism for myotube differentiation is claimed by decreasing G_{M3} ganglioside associated with the cytoskeleton leading to the alteration of cytoskeletal functions (Akita 1997; Sato 1997; Fanzani 2003). However, the exact function of Neu2 in cells and tissues is obscure and there is no identified genetic disorder associated with the deficiency of Neu2.

1.1.3. Neuraminidase 3

Neu3 is expressed from the gene Neu3 which is located on human chromosome 11q13.5 (Wada et al. 1999). It plays role in the caveolae microdomains of plasma membranes (Wada et al. 1999; Wang et al. 2002). It has a high expression levels in skeletal muscle, testis, adrenal gland and thymus and low expression levels in kidney, placenta, brain and lung (Wada et al. 1999). Neu3 is responsible for modulating oligosaccharide chains of gangliosides on lipid bilayer during transformation, differentiation and formation of cell interactions (Kopitz 1996; Kopitz 1998), being active against gangliosides that play role in signal transduction (Schneider-Jakob 1991). It has been shown that up-regulation of Neu3 is essential for cancer cell survival. However study with siRNA implicate that Neu3 is important player against cancer progression. Neu3 siRNA could be therapeutic agent providing apoptosis of cancer cells (Miyagi 2008).

1.1.4. Neuraminidase 4

Sialidase Neu4 enzyme is expressed from the gene Neu4 that maps in the telomeric region of the long arm of human chromosome 2 (2q37). Neu4 gene was discovered as a result of sequence database search that revealed homology to the human cytosolic sialidase Neu2 gene. Entire Neu4 gene is 6663 bp located in the position 239447224 – 239453886 of chromosome 2q37.3 and has four exons. Sialidase Neu4 enzyme has the highest expression level in liver and it is also ubiquitously expressed in all CNS (central nervous system) districts, colon, small intestine, kidney, heart, skeletal

muscle and placenta (Monti et al. 2004) and its expression is decreased during monocyte to macrophage differentiation (Stamatos et al. 2005). Sialidase Neu4 enzyme has two major isoforms; short form containing 484 amino acids with a molecular weight of 51.57 kDa (Monti et al. 2004) and long form containing 496 amino acids (Bigi et al. 2009) and additionally 18 transcripts related to sialidase Neu4 enzyme has been listed as in Ensemble database. It has a F/YRVP sequence motif and two classical Asp blocks. Sialidase Neu4 enzyme has been firstly characterized as a member of lysosomes and targeting of sialidase Neu4 enzyme to lysosomes by the mannose 6-phospate receptor has also been shown in literature (Monti et al. 2004). It has also been shown that sialidase Neu4 enzyme localizes in lysosomal lumen as a soluable hydrolase (Seyrantepe et al. 2004). In contradiction to these knowledge, it has been suggested that sialidase Neu4 enzyme long form localizes in mitochondria and sialidase Neu4 enzyme short form is associated with the endoplasmic reticulum (Bigi et al. 2009). Sialidase Neu4 enzyme has a broad substrate specificity almost equally against glycoproteins (mucin), oligosaccharides (sialyllactose) and sialylated glycolipids (mixed bovine gangliosides). It shows sialidase activity on synthetic substrates 2'-(4-methylumbelliferyl)-alpha-D-N-acetylneuraminic acid (4-MU-NANA or 4MU-NeuAc) at acidic pH 3.2 (Monti et al. 2004). An additional function of sialidase Neu4 enzyme can be explained as lysosomal catabolism of sialylated glycoconjugates with a supporting evidence showing degradation of storage materials from lysosomes of sialidosis and galactosialidosis patients. Overexpressed sialidase Neu4 enzyme activity in sialidosis fibroblasts has been shown with the clearance of accumulated substrates and normal morphological phenotype of the lysosomal compartment. In addition to this data, complete elimination of storage materials in 55% of sialidosis and 25% of galactosialidosis cells was achived by 3–5% of Neu4-expressing cells indicating the therapeutical potential of sialidase Neu4 enzyme in sialidosis and galactosialidosis for enzyme replacement therapy. *In vivo* studies reveal that $Neu4^{-/-}$ mice show vacuolization and lysosomal storage in lung and spleen cells. $Neu4^{-/-}$ mice also have increased level of G_{D1a} ganglioside and decreased level of G_{M1} ganglioside in brains as a supporting evidence of sialidase Neu4 enzyme desialylation activity against brain gangliosides (Seyrantepe et al. 2004). It has been shown that Neu4 is downregulated in human colon cancer cells and overexpression of Neu4 in cultured cells accelerates apoptosis and decreases invasiveness and motility (Miyagi 2008).

1.2. Lysosomes

Lysosomes are digestive organelles in cells. Lysosomes are membrane-bound compartments that contain more than 50 acid hydrolases processing catabolism reactions of the cell at an acidic pH of 4.6-5 (Nilsson et al. 2003). Function of lysosomes is basicly degradation of macromolecules like proteins, polysaccharides, nucleic acids, glycoconjugates and phospholipids. These substances are transported to lysosomes via endocytosis, phagocytosis, autophagy or direct transport. Degraded products can be released from lysosomes via diffusion or transport systems and after their release, they can be utilized to build new macromolecules or they can be used to produce metabolic energy (Suzuki 1994). Lysosomes are classified as the primary lysosome, the secondary lysosome and the residual body according to their physiological functions. The primary lysosome is a membrane-bound compartment containing hydrolytic enzymes like phosphatase, glucuronidase, sulfatase, ribonuclease, and collagenase that were synthesized in rough endoplasmic reticulum. The secondary lysosome is the fusion of the primary lysosomes with membrane-bound vacuoles containing materials for degradation. Residual bodies contain undegradable or slowly degradable materials (Zhang et al. 2009). The transport of lysosomal enzymes and membrane protein to lysosomes is achieved via *trans* Golgi network. Transport vesicles that bud from *trans* Golgi network deliver them to late endosomes. Lysosomal enzymes are recognized with their marker called mannose-6-phosphate(M6P) groups that attached to N-linked oligosaccharides. M6P Receptor Proteins recognize these M6P groups and provide package of hydrolases in to clathrin-coated vesicles which releases from *trans* Golgi network and deliver materials inside to a late endosome (Alberts, Johnson and Lewis et al. 2002). Besides hydrolases, proteases called cathepsins are present in lysosome. Cathepsins are also classified as three groups according to their amino acid content in active sites; cysteine cathepsins, aspartyl cathepsins and serine cathepsins (Zhang et al. 2009). Cathepsins are responsible for protein degradation, antigen presentation, bone resorption, and hormone processing (Turk et al. 2000). Some cathepsins like Cathepsin B and Cathepsin L are very important for maturation and integrity of the post-natal central nervous system (CNS) as a result of the data obtained from cathepsin $B^{-/-}$ $L^{-/-}$ mice showed brain atrophy (Felbor et al. 2002). Glycosphingolipids are also digested in lysosomes. They contain a hydrophobic

ceramide moiety and an extracytoplasmic oligosaccharide chain. They are classified into two groups, first one is neutral glycosphingolipids which contain monoglycosyl-/oligoglycosylsphingoids and monoglycosyl-/oligoglycosylceramides, second one is acidic glycosphingolipids which contain sialosylglycosylsphingolipids(gangliosides), sulfatides, glycuronoglycosphingolipids, phospho- and phosphonoglycosphingolipids (National Library of Medicine, 2011). They are distributed in nature from bacteria's cell membrane to man's cell membrane and are the major glycans found in vertebrate brain.

1.3. Lysosomal Storage Disorders

Lysosomal Storage Disorders (LSDs) are a group of nearly 50 rare diseases characterized as accumulation of waste materials due to deficiency of some enzymes, activators, transporters resulting large lysosomes in cells. LSDs have a wide spectrum of clinical phenotypes in accordance with the age of onset, severity of symptoms and central nervous system manifestation. The type of accumulated material and its tissue distribution, genetic backgrounds and environmental factors determine the severity of a lysosomal storage disorder. LSDs are inherited autosomal recessively but Fabry disease and Hunter syndrome are inherited X-linked recessively. Patients with this disease are born healty but sympthoms become visible progressively. Pathophysiology of LSDs include developmental delay, movement disorders, seizures, dementia, deafness and blindness. In addition some LSDs show hepatomegaly, splenomegaly and cardiac problems. The prevelance of LSDs are suggested as 1/8000 (Berg 2005). LSDs are classified as i) defects in glycan degradation, ii) defects in protein degradation, iii) defects in lysosomal transporters, iv) defects in lysosomal trafficking and v) defects in lipid degradation in five groups according to their causes as shown in Table 1.1. In the group of defects in lipid degradation, there are such diseases like Fabry disease, Farber disease etc. and one of these disorders is Tay-Sach Disease.

1.4. Tay-Sachs Disease

Tay-Sachs disease is one of the sphingolipid degradation disorder and characterized as accumulation of G_{M2} ganglioside due to the deficiency of beta-hexosaminidase A enzyme. Beta-hexosaminidases are responsible for degrading amino-hexose moieties containing beta-glycosidic bonds from terminal part of glycoproteins, proteoglycan and glycolipids (Mark et al. 2003). This disease causes neurodegeneration including developmental arrest, progressive neurological deficits and a shortened life span (Kolodny 1966). This group of condition contains two main disorders called Tay-Sachs disease and Sandhoff disease (Jeyakumar 2002). Tay-Sachs disease is an autosomal recessive disorder caused by the defects in alpha-subunit of the beta-hexosaminidase A that has 2 different subunits (one alpha and two beta) (Okada and O'Brien, 1969) resulting G_{M2} ganglioside accumulation in neuronal cells (Gravel et al. 1995).

Table 1.1. Lysosomal Storage Disorders

Disorder	**Primary deficiency (secondary deficiency)**	**Substrate**
Disorders of sphingolipid degradation		
Fabry disease	Alpha-galactosidase	Gal-Gal-Glu-ceramide
Farber disease	Ceramidase	Ceramide
Gaucher disease	Glucocerebrosidase	Glucosylceramide
G_{M1} gangliosidosis	beta-galactosidase	GMI ganglioside
G_{M2} gangliosidosis		
Tay-Sachs disease	**beta-hexosaminidase A**	**G_{M2} ganglioside**
Activator deficiency	**G_{M2} activator**	**G_{M2} ganglioside**
Sandhoff disease	**beta-hexosaminidase A and B**	**G_{M2} ganglioside**
Krabbe disease	Galactosylceramidase	Galactosylceramide
Metachromatic leukodystrophy	Arylsulfatase A	
Enzyme-deficient form	Saposin	Galactosylsulfatide
Activator-deficient form	(Ganglioside sialidase)	Galactosylsulfatide
Mucolipidosis IV	(Deficiency of all sulfatases)	
Multiple sulphate deficiency	Sphingomyelinase	(sulfatase substrates)
Niemann-Pick disease	Alpha-N-acetylgalactosaminidase	Sphingomyelin
Schindler disease		a-galNAc glycolipids

G_{M2} ganglioside is catabolized with the combination of beta- hexosaminidase A and G_{M2} activator that makes substrate accessible and soluble for beta- hexosaminidase A (Meier et al. 1991). The *in vivo* studies reveal various substrates accumulated in G_{M2} gangliosidosis (Sandhoff et al. 1989; Sandhoff 1991) as shown in Table 1.2.

Several variants of Tay-Sachs disease can be listed as beta1 variant, pseudo-deficiency, infantile, juveline and adult Tay-Sachs disease. Infantile Tay–Sachs Disease is the most common variant of the disease. Patients with infantile Tay–Sachs disease have zero activity of HexA and are affected by the disease in the first few months of their life showing neurodevelopmental delays and deficits like being less responsiveness to the environment, low facial expression, seizures, blindness, disability of crawling and standing, cherry-red spot in both optic fundi causing early death of the patient (Sachs 1887; Gravel et al. 1995).

Table 1.2. Storage of GSL in the G_{M2} gangliosidosis
(Source: Mahuran 1999)

	G_{M2} (nmol/g)	**G_{D2} (nmol/g)**	**G_{D1a} (nmol/g)**	**GalNac-G_{D1a} (nmol/g)**	**G_{A2} (nmol/g)**	**Gb4b (nmol/g)**
Control	20	40	500	2	0	180
Tay-Sachs	10000	80	400	30	1000	400
AB-variant	18000	90	200	2	4000	200
Sandhoff	9000	100	300	10	5000	2000

The autopsies of the patients provide knowledge about the disease's pathology having severe cerebral and cerebellar atrophy, neuronal degeneration (Johnson et al. 1980), ballooned neurons and neuronal loss (Moriwaki et al. 1977). Juvenile Tay–Sachs Disease appears between the ages of 3 - 5 following with ataxia, deterioration of activities, worsening motor functions and spasticity (Brett et al. 1973; Specola et al. 1990) generally causing death between the ages of 15-20 (Nardocci et al. 1992). Adult Tay–Sachs Disease is late onset form of the disease and appears at the age of nearly 18 with slowly progressing neurodegeneration (Neudorfer et al. 2005) with ataxia, gait disturbances, weakness, proximal muscle wasting, cramps, and fasciculations (Harding, Young, and Schon 1987; Federico 1987) and these patients do not present cherry-red spots. Beta-1 variant of the disease include a mutant HexA similar to wild-type HexA,

caused by the mutation in nucleotide 533 (G→A substitution) resulting an amino acid change (R178H) in the protein (Ohno and Suzuki, 1988) and present a phenotype including megalencephaly and macular cherry-rod spot (Grosso et al. 2003). Patients with pseudo deficiency show low HexA activity *in vitro* but no G_{M2} ganglioside accumulation or disease symptoms (Triggs-Raine et al. 1992). AB-variant of G_{M2} gangliosidoses is caused by the mutations in the GM2A gene that produces G_{M2} activator which is responsible for proper function of beta-hexosaminidase. Sympthoms of this disease are inability of sitting, crawling, seizures, vision and hearing loss, mental retardation, and paralysis (OMIM 2011; Mahuran 1999). Sandhoff disease is also a lipid storage disorder caused by deficiency of functional beta-hexosaminidase A and B (Sandhoff et al. 1968). Major stored materials are G_{M2} ganglioside and oligosaccharides (Sandhoff et al. 1989). The main reason of deficiency is mutations in HEXB gene (Gomez-Lira et al. 1995). Clinical features are very similar to Tay-Sachs disease including blindness, progressive mental and motor deterioration, cherry red spots and macrocephaly. Patients die by the age of 3 years (OMIM, 2011).

The mouse models of Tay-Sachs disease has been generated by the targeted disruption of the HexA gene. Analysis of these knock-out mice revealed that although G_{M2} ganglioside accumulated in the brain, mice did not present any kind of human Tay-Sachs phenotype like behavioural or motor abnormalities even at the age of 1 year. Explaination of this situation is that $HexA^{-/-}$ mice possess a lysosomal neuraminidase sufficiently and they can convert G_{M2} to G_{A2} ganglioside and lactocyl ceramide by HexB isoenzyme as shown in the Figure 1.1 (Cohen-Tannoudji et al. 1995; Phaneuf 1996; Sango 1995).

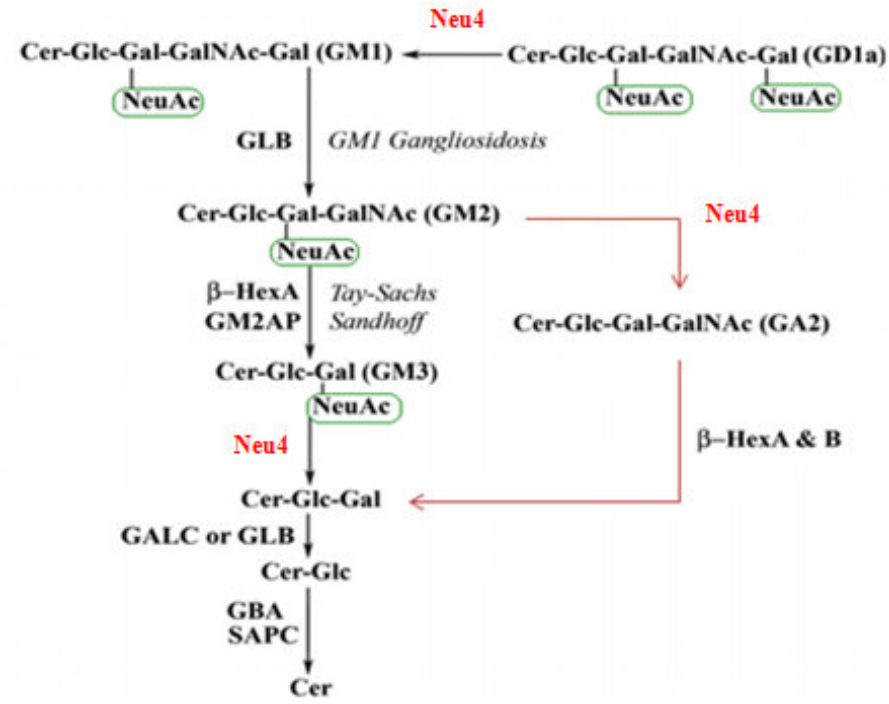

Figure 1.1. Possible by-pass mechanism of sialidase Neu4 enzyme shown with red arrows. GLB, beta-galactosidase; GM2AP, GM2 activator protein; GBA, acid beta-glucosidase; SAPC, sapocin C (Adapted from: Seyrantepe et al. 2008)

Mice can convert G_{M2} to G_{A2} with their neuraminidase whereas human may not because of low abundancy of neuraminidase. Targeted disruption of HexA in mice prevented conversion of G_{M2} to G_{M3} ganglioside like in Tay-Sachs patients. It has been shown that mice fibroblasts convert G_{M2} to G_{A2} which is a asialo-G_{M2} produced by a sialidase, instead of converting to G_{M3} (Phaneuf 1996; Sango 1995). This metabolic by-pass prevent mice to present phenotype of Tay-Sachs disease.

Mice double-deficient in $HexA^{-/-}$ and $Neu4^{-/-}$ have been generated and epileptic crisis, degenerating neurons, motor impairment like tremor, weakness, spasticity and additionally G_{M2} ganglioside accumulation have been observed in contrast to single knock-out $HexA^{-/-}$ or $Neu4^{-/-}$ mice. This data reveals that due to sialidase Neu4 enzyme deficiency, $HexA^{-/-}$ mice show a severe phenotype supporting the modifier role of sialidase Neu4 enzyme in the metabolic by-pass of Tay-Sachs disease mouse model. The increase in disease severity in double deficient mice indicate that sialidase Neu4 enzyme is not the only responsible enzyme for by-pass mechanism. All these data suggest that sialidase Neu4 enzyme might be a potential therapeutic modifier by a pharmacologic induction through its upregulation with an agent in the future (Seyrantepe et al. 2010).

1.5. Transcriptional Machinery

Healty development and maintenance of an organism needs correct spatial and temporal expression of its genes. This regulated expression is provided by the process of transcription. Transcription is the process of producing complementary RNA molecule using the DNA as a template (Narlikar and Ovcharenko 2009). Transcription begins with the binding of RNA Polymerase Complex to the DNA sequence called promoter in all organisms. RNA Polymerase Complex triggers transcription initiation followed by elongation of the transcript. Transcription is regulated in two different ways; the promoter level (cis-regulation) and the RNA Polymerase level (trans regulation). These regulatory mechanisms are different among prokaryotes and eukaryotes.

Eukaryotic organisms have different types of tissues and cells. Proper function of these cells is maintained by the process called transcriptional regulation that includes correct expression of thousands of genes in the organism. This regulation can be achived by transcription factors that bind specifically to the DNA sequences called transcription factor binding sites. Generally different types of transcription factors function together providing the regulation of excess numbers of protein coding genes. The transcript of the desired gene is transcribed by RNA Polymerase II that recognize the transcription start site (TSS) with the existence of the general transcription factors (GTFs). A special DNA region called core promoter presents TSS and other binding sites for different subunits of GTFs to form a complex and provides binding of RNA Polymerase to this assembly to form transcription initiation complex (TIC). The eukaryotic transcription machinery can be classified into two groups; trans-acting elements that are DNA binding proteins and cis-acting elements that are specific DNA regions (Narlikar and Ovcharenko 2009).

Trans-regulatory elements constitute transcription factors (TFs). Transcriptional factors contain two major domains; a DNA-binding domain and a transcription regulation domain (Mitchell 1989). The DNA binding domain is responsible for recognizing the specific DNA sequence and binding of the TFs to this sequence. The transcription regulation domains are responsible for binding transcriptional regulatory proteins to the TF to form functional complexes (Kummerfeld 2006). It is suggested that 10% of the gene products in human genome are candidate transcription factors,

nearly 1400 TFs (Vaquerizas 2009) and the number of candidate transcription factors might be related to the genome size and organism complexity proportionally (van Nimwegen 2003). Main functions of trans-regulatory elements are gene activation and gene repression (Narlikar and Ovcharenko 2009). Both mechanisms include DNA-binding proteins and non-DNA binding proteins. Gene activation is mediated with binding of DNA-binding proteins (activators) to a region of 5-15 bp DNA sequence (Bulyk 2003). Activators can bind to the core promoter that places in proximal promoter region and 5' untranslated region (UTR) and they can also bind to enhancer that place in distal promoter region resulting activation of transcription due to TIC attraction (Khoury 1983). Non-DNA binding proteins (co-activators) are responsible to combine GTFs and activators to trigger TIC formation (Narlikar et al. 2002). Gene repression also includes DNA-binding proteins (repressors) and non-DNA binding proteins (co-repressors) as mentioned before. DNA-binding proteins (repressors) may inhibit transcription by competing with activator, interfering the activator's activity due to close proximity, inhibiting TIC formation as a result of binding to silencers, causing lack of communication due to binding to insulators. Nucleosomes are also classified as repressors for their ability to prevent incorrect transcriptions (Kaplan 2003). Non-DNA-binding proteins (co-repressors) that do not bind to DNA directly may inhibit transcription via protein-protein interactions through blocking the binding of activators and TIC to DNA by reorganization of the chromatin structure, forming useless activator complex to bind DNA or co-activator, inhibiting TIC directly.

Transcription factors can not operate themselves. They co-operate with specific sequences of target genes (Madan 2003). These binding sites are called cis-regulatory elements and are classified according to their function or genomic location; promoters, enhancers, silencers and insulators as shown in Figure 1.2. Promoters are devided into two groups containing core promoter which is about 100 bp around the TSS (Carey et al. 2001) and proximal promoter which places a few base pair further from TSS (Maston 2006). Core promoter localizes the start of a gene and presents binding sites for GTFs and preinitiation complex (PIC). The core promoter elements are TATA box, an Initiator element (Inr), a Downstream Promoter Element (DPE), Downstream Core Element (DCE), a TFIIB-Recognition Element (BRE), and a Motif Ten Element (MTE) (Lim et al. 1990).

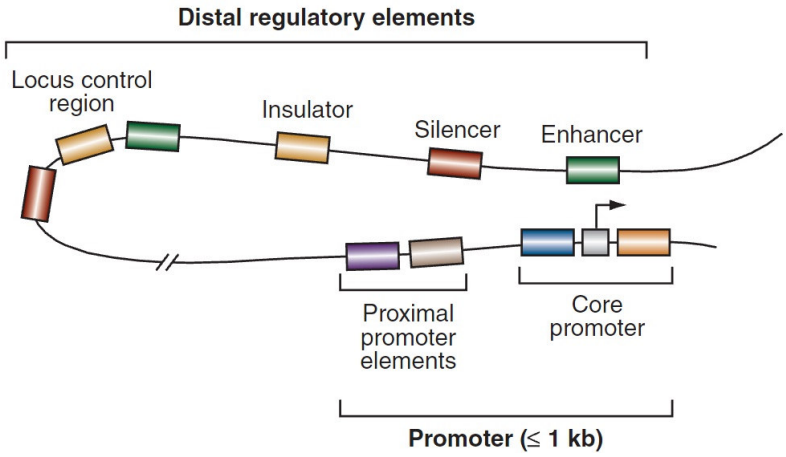

Figure 1.2. Mammalian regulatory elements
(Source: Glenn et al. 2006)

TATA box presents binding site for TBP (TATA binding protein) which is a subunit of TFIID, BRE is specifically recognized by TFIID. DPE interacts with TAF6 and TAF9, Inr interacts with TAF1 and TAF2, DCE interacts with TAF1 (Lee et al. 2005, Smale 2003). It is suggested that there might be undiscovered promoter structures like ATG deserts due to the absence of these core promoter elements in quarter of all promoters (Lee et al. 2005). The proximal promoter localizes close to upstream of core promoter and presents binding sites for activators. Enhancers localizes a greater distance from TSS harbouring the binding sites of multiple activators. They can be found in upstream, downstream or within an intron sequence of the gene (Blackwood 1998). There are two mechanisms related to enhancer activity, looping and DNA scanning. Looping theory includes binding of activators to enhancers enabling the loop-out between enhancer and the core promoter bringing activators close to the promoter. According to this theory promoter ensure correct gene activation (Carey et al. 2001). DNA scanning mechanism includes movement of activators along the DNA until they reach the correct target promoter as a result of enhancer binding (Blackwood 1998).

Silencers are sequence specific elements repressing the transcription of a gene functioning independently from the promoter. There are also some silencers reported as position-dependent manner. Silencers can be included in enhancers or they can function as independent modules containing repressor binding sites (Ogbourne 1998). Insulators are responsible for preventing a gene being affected from the other gene that has a transcriptional activity by decreasing the activity of transcriptional regulatory elements inside the certain domains. They function in two different ways, by inhibiting the

enhancer/silencer and promoter interaction or by stopping the spread of repressive heterochromatin (Gaszner 2006). There are also additional regulatory elements in human genome, locus control regions (LCRs) and matrix attachment regions (MARs). Locus control regions (LCRs) are responsible for regulation of a cluster of genes in some specific cell types (Li et al. 2002). MARs are suggested to play a role in changes in chromatin structure for accessibility to transcription factors (Hart and Laemmli 1998).

1.6. Transcriptional Regulatory Elements and Human Diseases

Cis-regulatory sequences are very important for healthy control of gene expression. Alteration in gene expression is an indicator of human disease susceptibility with a high heritable manner. Human genetic diseases caused by mutations in non-coding regulatory sequences increase rapidly. 1459 regulatory mutations were connected to 700 genes causing human-inherited disorders and 1% and 2% of these mutations harbour in noncoding regions of the genome majoring proximal and distal promoter elements according to Human Gene Mutation Database. These mutations have very significant morphological, physiological and neurological consequences (Epstein 2009) as listed in Table 1.3.

Table 1.3. Transcriotion regulatory elements and diseases
(Source: Glenn et al. 2006)

Regulatory Element	**Disease**	**Mutation (bound factor)**	**Affected Gene**
Core promoter	β-thalassemia	TATA box, CACCC box, DCE	β-globin
Proximal promoter	Charcot-Marie-Tooth disease Hemophilia Progressive myoclonus Epilepsy β-thalassemia δ-thalassemia	215 bp upstream of TSS CCAAT box (C/EBP) Expansion 70 bp upstream of TSS CACCC box (EKLF) 77 bp upstream of TSS (GATA-1)	connexin-32 factor IX cystatin B β-globin δ-globin
Enhancer	X-linked deafness	Microdeletions 900 kb upstream	POU3F4
Silencer	Asthma and allergies	509 bp upstream of TSS (YY1)	TFG-β
Insulator	Beckwith-Wiedemann syndrome	CTCF binding site (CTCF)	H19/Igf
LCR	α-thalassemia β-thalassemia	62 kb deletion upstream of gene cluster 30 kb deletion removing 5' HS2–5	α-globin genes β-globin genes

It has also been shown that mutations in components of the transcriptional machinery are related to human diseases listed in Table 1.4.

Table 1.4. Regulatory components and diseases
(Source: Glenn et al. 2006)

Component	**Disease**	**Mutated Factor**
General transcription factors	Xeroderma pigmentosum	TFIIH
Activators	Congenital heart disease Down syndrome with acute	Nkx2–5 GATA-1
Repressors	X linked autoimmunity-allergic	FOXP3
Coactivators	Parkinson's disease	DJ-1
Chromatin remodeling factors	Retinal degeneration Rett syndrome α-thalassemia myelodysplasia syndrome	ataxin-7 MeCP2 ATRX

1.7. Genetic Reporter Systems

Reporter genes have been preferred to investigate biological processes via their transcriptional regulation (Wood 1995). Reporter genes like β-galactosidase (*lacZ*), firefly luciferase (*luc*), bacterial luciferase (*luxCDABE*) and green fluorescent protein (*gfp*) have been used for detection of molecular events like receptor activity, transcription factors, intracellular signaling, mRNA processing, protein folding and it is also used to understand if a gene had been taken up into a cell/organism or expressed. Mechanism of this method includes the attachment of the reporter gene to a regulatory sequence and insertion of this structure into a biological system then obtaining a signal via its own expression (Wood 1995). The mostly used three reporter systems are β-galactosidase, luciferases and fluorescent proteins. β-galactosidase protein (β-gal) converts lactose to galactose and glucose. This enzyme is also capable of degrading such substrates like chromogens o-nitrophenol β-D-galactopyranoside (ONPG), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 3,4-cyclohexenoesculetin-β-D-galactopyranoside (S-gal) (James et al. 1996), giving yellow, blue and black products, respectively. Green fluorescent protein is the most common fluorescent protein used in reseach and it is functional in both eukaryotes and prokaryotes. The advantages of using fluorescent proteins are wide spectrum of hosts, absence of cell lysis or substrate addition. Different colors of variants like blue, yellow and red are available commercially (ClonTech Inc.). The disadvantage of this system can be explained that fluorescent proteins continue emitting fluorescence after host died and the fluorophore of the wild-type GFP protein should be renewed by biological processes (Heim et al. 1995; Katranidis 2009). Luciferases are the enzymes producing luminescence and are classified as eukaryotic or bacterial. Firefly luciferase (*luc*) is the most commonly preferred reporter gene (de Wet et al. 1987) having the benefits of high sensitivity, tight coupling of the Luc protein concentration with luminescence output, no requirement of post-translational modifications, immediate activity after translation (de Wet et al. 1985). Firefly luciferase (Photinus luciferin:oxygen 4- oxidoreductase, EC 1.13.12.7) was isolated from *Photinus pyralis*. It is a 61kDa monomeric protein that functions without post-translational processing. This enzyme converts beetle luciferin into oxyluciferin giving a photon emission as an by product shown in the Figure 1.3 (Wood et al. 1984; de Wet et al. 1985) and this light is detected with luminometers,

additionally it has an optimum pH of 7.8 (Steghens, Min and Bernengo 1998). Luciferases are generally preferred for transcriptional activity research (Fan and Wood 2007) in cells transfected with a plasmid containing a luciferase gene as an reporter driven with a candidate promoter as shown in Figure 1.3.

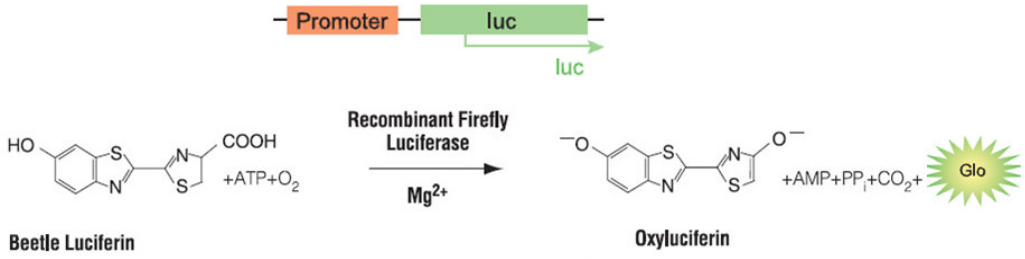

Figure 1.3. Catalytic reaction of luciferase
(Adapted from: Promega technical manuel; Fan and Wood 2007)

The disadvantage of this system is need for costly substrate, luciferin for monitoring and measuring.

1.8. Electrophoretic Mobility Shift Assay (EMSA)

Regulation of many cellular processes like DNA replication, recombination, repair, transcription are maintained by interaction of proteins with DNA. EMSA is based on slow migration of DNA:protein complexes which is caused by protein binding than free DNA molecules that are run on native polyacrylamide gel or agarose gel electrophoresis (Hendrickson 1985; Revzin 1989). This retarded DNA:protein complexes are called as shifts. Crude nuclear or whole cell extracts rather than purified proteins can be used as a source of DNA binding proteins. EMSA can be used as qualitatively by identifying DNA binding proteins that are specific to a given sequence with mutagenesis or identifying specific sequences of a gene's upstream region and as quantitatively by measuring thermodynamic and kinetic parameters of binding reactions (Fried et al. 1981; Garner et al. 1981; Fried et al. 1984; Fried 1989). Resolving ability is based on stability of DNA:protein complexes which is in fact unstable but maintained stabile with low ionic strenght of electrophoresis buffer during migration in the gel. Nucleic acids which can be a double-stranded DNA, RNA molecule used in EMSA can be labelled radioisotopes, fluorophores and biotin and these labels can be detected by

autoradiography, fluorescence imaging and chemiluminescent imaging, respectively (Rye et al. 1993; Forwood et al. 2006; Kang et al. 2005; Li et al. 2004). Binding of nonspecific proteins to the labeled target DNA is reduced by nonspecific competitor DNA which are repetitive polymers called as poly(dI·dC) or poly(dA·dT) that are responsible for adsorbtion of proteins that bind to any general DNA sequence to their nonspecific sites. Determining the specificity of DNA:protein binding is achieved with addition of 50, 100, 150 etc. excess of unlabelled DNA concentration for competition assays (Lane 1992).

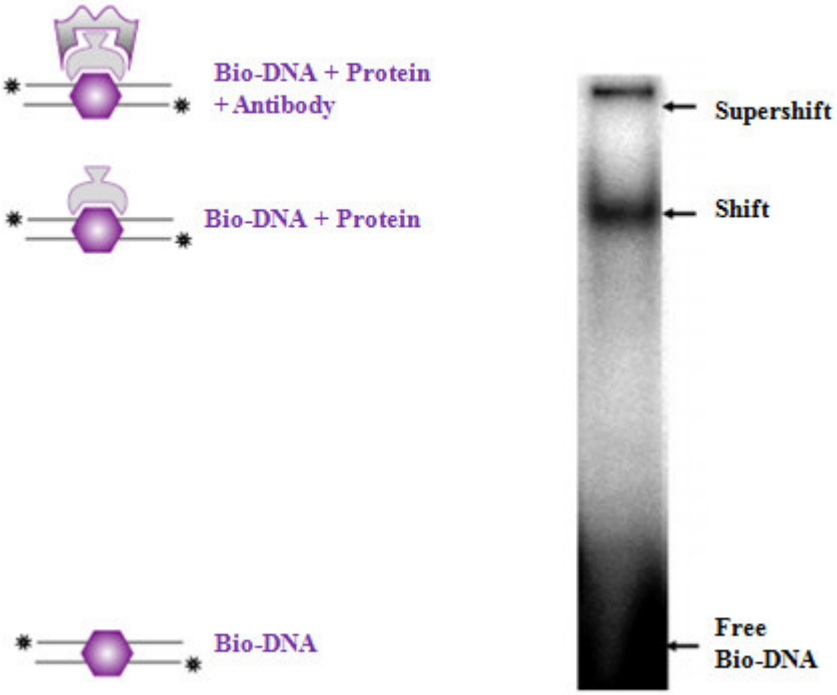

Figure 1.4. Electrophoretic mobility shift assay
(Adapted from: Biochem.arizona , 2011)

When an antibody which is specific to a transcription factor included in the binding reactions of DNA and proteins, antibody can bind specifically to the transcription factor that is bound to the DNA. This DNA:protein:antibody complex migrates more slowly than 'shift' and also free labelled DNA and called as supershift as shown in Figure 1.4 (Kristie 1986).

1.9. The Aim of the Study

The aim of the study is to identify promoter region of human sialidase Neu4 gene which has not been studied yet. We also aimed to show specific interactions of transcription factors in the promoter region.

CHAPTER 2

MATERIALS AND METHODS

2.1. Bioinformatic Analysis of Promoter Region for Neu4 Gene and Primer Design

3000 bp upstream region from Neu4 gene start codon (ATG) were considered to be a region containing the regulatory elements for Neu4 gene. The sequence of 3000 bp were obtained from NCBI (The National Center for Biotechnology Information). This sequence were input to TESS (Transcription Element Search System) for analysis and mapping of transcription binding sites in it. After obtaining the map of transcription binding sites, reverse and forward primers were designed according to different motifs as shown in Table 2.1 for further regulation studies using PrimerDesign3 tool (version 0.4.0, http://frodo.wi.mit.edu/primer3/).

2.2. Primers

Primers were designed to obtain seven different DNA fragments between 187 bp and 3011 bp according to their transcription factor binding sites as shown in the Figure 2.1. These primers also included restriction sites at their 5' ends for cloning into reporter vectors during further assays. That's why reverse primer which is shared for all forward primers during PCR contain a NheI restriction site (GCTAGC) and forward primers contain a KpnI restriction site (GGTACC) as shown below.

Table 2.1. Reverse and Forward primers

Primers	**Sequence**
F1	5'- **GGTACC**CTCCTGGGTGCCCATCTG- 3'
F2	5'- **GGTACC**TCTCTGGAGCAGCAGACC- 3'
F3	5'- **GGTACC**GTGAATGGGACTGGCAGGAG- 3'
F4	5'- **GGTACC**GGGGGAAGCTTTCCTTAACC- 3'
F5	5'- **GGTACC**ACACCCTGGCCAGACAGC- 3'
F6	5'- **GGTACC**CCTGTCCCTGAGCGGAAC- 3'
F7	5'- **GGTACC**CTCCGTGTCAGTGTGCATTC- 3'
Reverse primer	5'- **GCTAGC**GCTGCAGAGCTCATCATGG- 3'

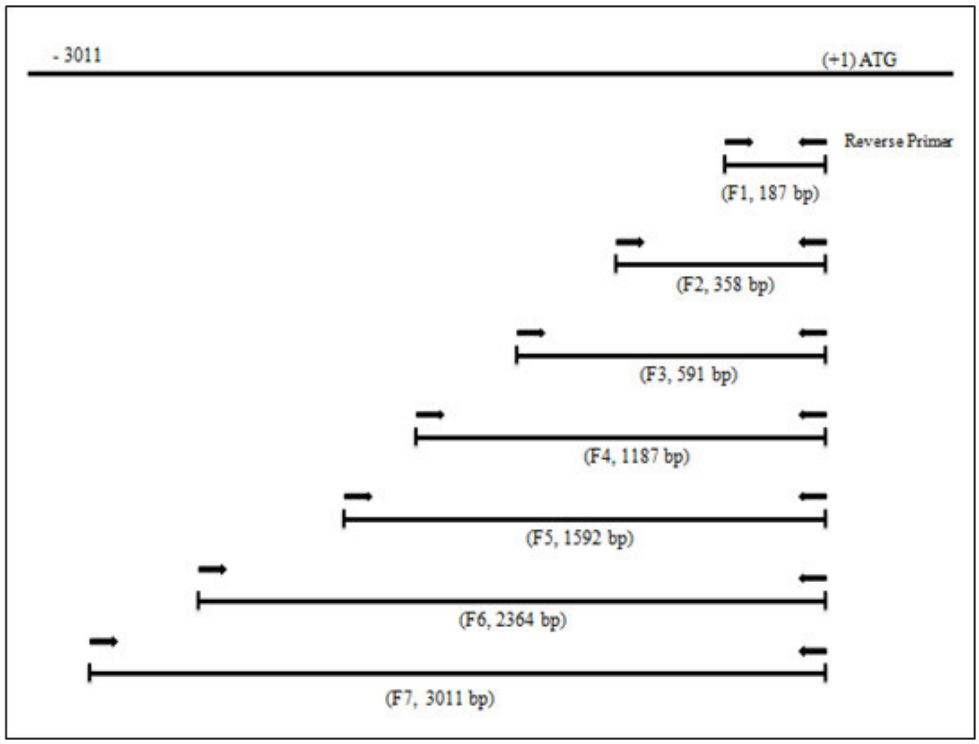

Figure 2.1. Primers amplifying seven different DNA regions

2.3. Plasmids

There are three different plasmids used in this study. These plasmids can be listed as pCR 2.1 TOPO TA cloning vector, pGL 4.12 Firefly vector and pGL 4.74 Renilla vector shown in the Figure 2.2 - 2.3 - 2.4 .

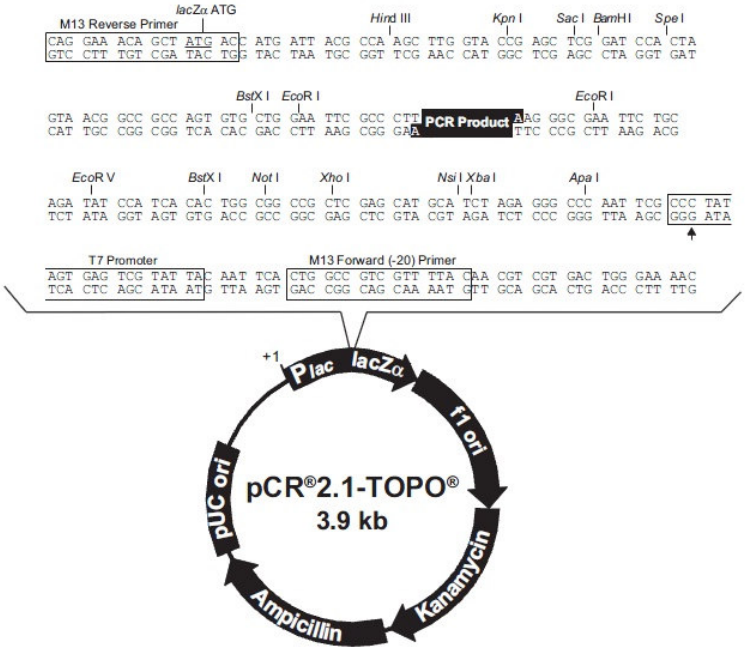

Figure 2.2. pCR 2.1 TOPO vector(Invitrogen)

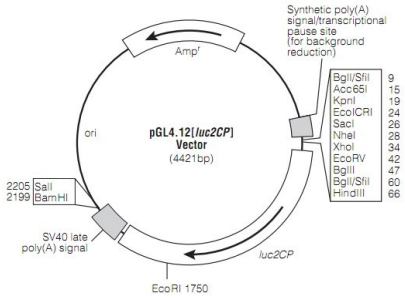

Figure 2.3. pGL 4.12 vector (Promega)

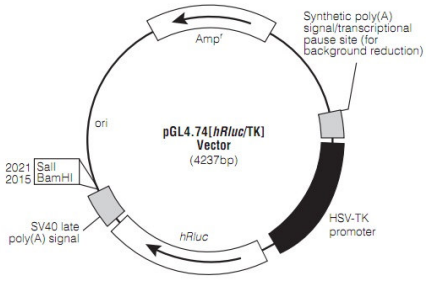

Figure 2.4. pGL 4.74 vector (Promega)

2.4. Commercial Kits

Table 2.2. Commercial kits

Kits	Supplier Company
Wizard® Genomic DNA Purification Kit	Promega,
i-Taq DNA Polymerase	Intron Biotechnology
Genejet Gel Extraction Kit	Fermentas
TOPO TA Cloning Kit	Invitrogen
GeneJET™ Plasmid Miniprep Kit	Fermentas
PureLink™ Plasmid DNA Purification Kits	Invitrogen
GeneJET™ PCR Purification Kit	Fermentas
TurboFect™ in vitro Transfection Reagent	Fermentas
Dual-Luciferase® Reporter Assay System	Promega
NE-PER® Nuclear and Cytoplasmic Extraction Reagents	Thermo Scientific
Pierce® BCA Protein Assay Kit	Thermo Scientific
Biotin 3' End DNA Labeling Kit	Thermo Scientific
LightShift® Chemiluminescent EMSA Kit	Thermo Scientific

2.5. DNA Isolation, PCR Amplification of Seven Different Fragments

Human DNA were isolated from blood with Wizard® Genomic DNA Purification Kit (Promega, A1125) according to manufacturer's instructions. This DNA was used in Polymerase Chain Reaction (PCR) with i-Taq DNA Polymerase (Intron Biotechnology, 25022). Seven different fragments were amplified with specific primers designed before in the mixtures and conditions as shown in Table 2.3 and Table 2.4, respectively.

After DNA fragments amplified with PCR, they were run in 1% agarose gel. Fragments were purified using Genejet Gel Extraction Kit (Fermentas, K0513) from 1% agarose gel.

Table 2.3. PCR mixture

Components	Volume (µl)
dH_2O	40
10X Buffer	2
DMSO	2.5
dNTPs (2,5 mM each)	2
Reverse Primer (100 pmol)	1
Forward Primer (100 pmol)	1
Template DNA (70 ng/µl)	1
i-Taq DNA Polymerase(5 U/µl)	0.5
Total Volume	50

Table 2.4. PCR conditions

Cycle Number	Cycle	Temperature(^oC)	Duration
1	Initial Denaturation	94	2 minutes
30	Denaturation	94	20 – 30 seconds
	Annealing	58 – 65	30 seconds – 1 minute
	Elongation	68	45 seconds– 2 minutes
1	Final Elongation	72	5 – 10 minutes

2.6. TA Cloning and Confirmation with Restriction Enzyme Digestion

Purified DNA fragments were cloned with TOPO TA Cloning Kit (Invitrogen, K4520-01) according to manufacturer's instructions. One Shot® MAX Efficiency® DH5 α -$T1^R$ Chemically Competent *E. coli* cells (Invitrogen, 12297-016) were used for bacterial transformation. White colonies were selected by blue-white screening. These selected colonies were inoculated into LB-broth medium containing 100 µg/ml

kanamycin and incubated at 37 ^{o}C in a shaker incubator for overnight. Plasmids were purified using GeneJET™ Plasmid Miniprep Kit (Fermentas, K0502). Purified plasmids were digested with EcoRI restriction enzyme at 37 ^{o}C for 2 hours for confirmation of the TA cloning as shown in Table 2.5.

Table 2.5. Digestion Mixture

Component	**Volume (µl)**
dH_2O	15.25
10X Buffer	2.0
Plasmid (0.5 µg)	2.5
EcoRI (10 u/µl)	0.25
Total volume	20

All digestion mixtures were run on 1% agarose gel for detection of confirmation.

2.7. Sequence Analysis of the Cloned Fragments and BLAST Search

Cloned TOPO plasmids containing seven different fragments were sequenced at Izmir Institute of Technology, Biotechnology and Bioengineering Research and Application Center as a service provider by different fluorescent dye labelled dideoxynucleotide chain terminating method. Cloned TOPO vectors were sequenced with both M13 primer and T7 primer. The sequences obtained from the sequencer were analysed using BLAST (Basic Local Alignment Search Tool).

2.8. Propagation and Confirmation of Firefly and Renilla Plasmids

pGL 4.12 (Firefly) and pGL 4.74 (Renilla) plasmids were kindly provided from Prof. Dr. Bünyamin Akgül from Izmir Institute of Technology, Department of Molecular Biology and Genetics. These plasmids were propagated as midipreps with PureLink™ Plasmid DNA Purification Kit (Midiprep, Invitrogen, K2100-04). These plasmids were confirmed using PstI and AccI at 37 ^{o}C for 16 hours for pGL4.12 and AlwNI and BalI at 37 ^{o}C for 16 hours for pGL 4.74. Digestion mixtures were loaded on 1% agarose gel and run.

2.9. Cloning of the Fragments into pGL 4.12 Firefly Vector

Cloning efficiency is highly related to formation of sticky ends at the ends of vector and inserts. Enzyme units and buffer selection were determined as a recommendation of Fermentas Double Digestion Tool (http://www. fermentas.com /en/tools/doubledigest). We double digested pGL4.12 with NheI and KpnI separately. KpnI was used for first digestion for 1 hour at 37 ^{o}C in KpnI buffer and then KpnI was denatured at 80 ^{o}C for 20 minutes. NheI was added into the digestion mixture and incubated at 37 ^{o}C for 1 hour more.

At the end of the incubations, digestion mixture was purified using GeneJET™ PCR Purification Kit (Fermentas, K0701) and its concentration was determined as 0.5 ng/µl. Separately digested pGL 4.12 was used for cloning of all seven fragments. TOPO vectors including our DNA inserts were digested with KpnI and NheI at 37 ^{o}C for 2 hours at the same time. At the end of the digestions, all mixtures were run on 0.8 % agarose gel and inserts were extracted with Genejet Gel Extraction Kit (Fermentas, K0513) and their concentration were measured with nanodrop (NanoDrop Technologies, Inc. ND-1000 spectrophotometer). Purified inserts were used in ligation with double digested pGL 4.12 with T4 DNA Ligase (Invitrogen, 15224-017) according to manufacturer's instructions.

After ligation, 2 microliters of each ligation mixture were transformed into One Shot® MAX Efficiency® DH5 α -T1^{R} Chemically Competent *E. coli* cells (Invitrogen, 12297-016) according to manufacturer's intructions and bacterial cells were plated on 100 µg/ml Ampcillin LB-agar plates following 16 hours incubation at 37 ^{o}C. All

transformations produced colonies on the plates and they were inoculated into 100 μg/ml Ampcillin LB-broth medium for 16 hours at 37 ^{o}C. After overnight incubation, cloned pGL 4.12 vectors were isolated from bacterial cultures with GeneJET™ Plasmid Miniprep Kit (Fermentas, K0502). These recombinant plasmids were confirmed by double digestion with NheI and KpnI at 37 ^{o}C for 2 hours. Double digestions showed that all seven fragments were cloned into pGL 4.12 firefly vector. Due to correct cloning of fragments as a result of double digestions, large scale plasmid production was performed by inoculating miniprep bacterial cultures into nearly 50 ml LB-broth containing 100 μg/ml Ampicillin incubating at 37 ^{o}C for 16 hours and isolating with PureLink™ Plasmid DNA Purification Kit (Invitrogen, Midiprep K2100-04).

2.10. Transfection of Luciferase Vectors into HeLa Cells and Optimization of Dual Luciferase Reporter (DLR) Assay System

HeLa cells were maintained in T25 tissue culture flasks in their growth medium called DMEM medium that contains 20% FBS, 1% Penicillin, 2mM L-Glutamine. These cells were transferred into 96-well plates for transfection assays. Fermentas Turbofect in vitro transfection reagent was used for all of the transfections according to manufacturer's instructions. For optimization of DLR assay, different ratios of pGL 4.12 and pGL 4.74 vectors and different incubation times were investigated. Ratios of 10:1 (pGL 4.12 : pGL 4.74) , respectively and 30:1(pGL 4.12 : pGL 4.74) , respectively were investigated for both 24 hours incubation and 48 hours incubation in duplicate based on literature knowledge. Firstly, HeLa cells were passaged from their growth medium to 96-well plate one day before to become fresh and to form 90% confluency prior to transfection. Transfection mixtures of 10:1 ratio and 30:1 ratio for a single well of 96-well plate were prepared as shown in the Table 2.6 below and incubated at room temperature for 20 minutes.

Table 2.6. Transfection mixtures for optimization

Component	Ratio	
	10 : 1	**30 : 1**
DMEM Blank	20 µl	20 µl
Turbofect	0.4 µl	0.4 µl
pGL 4.12 (100 ng/µl)	2 µl (10X)	2 µl (30X)
pGL 4.74 (20 ng/µl)	1 µl (1X)	0.35 µl (1X)

These different ratios were examined for optimum Firefly and Renilla luminescence. Ratio of 10:1 were examined for both 24 hours and 48 hours as the ratio of 30:1 were examined seperately from each other. DLR assay was performed with reagents in the kit according to manufacturer's instructions.

2.11. Luminometrical Measurement of Seven Fragments

Seven DNA regions that were cloned into pGL 4.12 were used as test samples in this assay and pGL 4.74 was used for normalization of DLR assay. Empty pGL 4.12 was also used as negative control with pGL 4.74. In 96-well plates, three wells were prepared as 'Blank' which has only cultivated HeLa cells and standard growth medium without any transfected plasmids, three wells were prepared as negative control which includes HeLa cells co-transfected with both empty pGL 4.12 and pGL 4.74, twenty-one wells were prepared as test samples co-transfected with seven different DNA regions cloned into pGL4.12 and pGL 4.74. As it can be inferred from above, all transfections for each DNA region were performed on the same day as triplicates. For a meaningful scientific conclusion, at least three independent experiments were performed on different days with three blanks, three negative controls and twenty-one independent test samples.

2.12. Electrophoretic Mobility Shift Assay (EMSA)

Nuclear proteins were isolated from HeLa cells using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, 78833). Concentration of isolated proteins were determined using Pierce® BCA Protein Assay Kit (Thermo Scientific, 23225). Biotinilation of F1 fragment (187 bp), F2 fragment (358 bp), F3 fragment (591 bp) was performed using Biotin 3' End DNA Labeling Kit (Thermo Scientific, 89818). EMSA was performed using LightShift® Chemiluminescent EMSA Kit (Thermo). Binding reactions were run on 3,5 % native-polyacrylamide gel and electroblotted to positively charged membrane (Pall Coorporation). Chemiluminescence was detected with Nucleic Acid Detection Module (Thermo Scientific, 89880). For 187 bp, 358 bp and 591 bp DNA regions, EMSA binding reactions were set as listed in Table 2.7. For supershift assay; binding reaction of c-myc antibody is shown in Table 2.8

Table 2.7. EMSA mixtures for 187 bp upstream region(F1), 358 bp upstream region(F2), 591 bp upstream region(F3) of Neu4 gene.

Component (µl)	**F1 DNA fragment**	**F1 DNA fragment + Nuclear Protein**	**F1 DNA fragment + Nuclear Protein + Unlabelled DNA**	**F2 DNA fragment**	**F2 DNA fragment + Nuclear Protein**	**F2 DNA fragment + Nuclear Protein + Unlabelled DNA**	**F3 DNA fragment**	**F3 DNA fragment + Nuclear Protein**	**F3 DNA fragment + Nuclear Protein + Unlabelled DNA**
dH_2O	16	15	14	16	15	14	16	15	12
10X Buffer	2	2	2	2	2	2	2	2	2
Salmon Sperm (1µg/µl)	1	1	1	1	1	1	1	1	1
Unlabelled DNA	-	-	1	-	-	1	-	-	3
Nuclear protein (5 µg/µl)	-	1	1	-	1	1	-	1	1
Labelled DNA (5 pmol)	1	1	1 (preincubation at room temperature for 5 min.)	1	1	1 (preincubation at room temperature for 5 min.)	1	1	1 (preincubation at room temperature for 5 min.)
Total volume	20	20	20	20	20	20	20	20	20

Table 2.8. EMSA mixtures for 187 bp upstream region(F1) and supershift assay.

Component (µl)	**F1 DNA fragment**	**F1 DNA fragment + Nuclear Protein**	**F1 DNA fragment + Nuclear Protein + c-myc antibody**	**F1 DNA fragment + Nuclear Protein + Unlabelled DNA**
dH_2O	16	15	10	14
10X Buffer	2	2	2	2
Salmon Sperm (1µg/µl)	1	1	1	1
Unlabelled DNA	-	-	-	1
Nuclear protein (5 µg/µl)	-	1	1 (Nuclear protein) + 5 (c-myc)	1
Labelled DNA (5 pmol)	1	1	1	1 (preincubation at room temperature for 5 min.)
Total volume	20	20	20	20

CHAPTER 3

RESULTS

3.1. Bioinformatic Analysis of Promoter Region for Neu4 Gene and Primer Design

3000 bp upstream region of Neu4 gene was obtained from (The National Center for Biotechnology Information) and this sequence was analysed using TESS (Transcription Element Search System). Regulatory elements were illustrated in Figure 3.1 as a result of TESS predictions. Primers that can amplify seven different regions were designed according to these regulatory elements to investigate their different effects.

Figure 3.1. Regulatory elements in 3000 bp DNA region illustrated in different colors

3.2. DNA Isolation and PCR Amplification of Seven Different Fragments

DNA isolated from blood and its concentration measured as 70 ng/ µl using NanoDrop ND-1000 Spectrophotometer. Seven different DNA regions were amplified using specific primers designed before and run on 1% agarose gel as shown in Figure 3.2 and purified from 0,8 % agarose gel using Genejet Gel Extraction Kit (Fermentas).

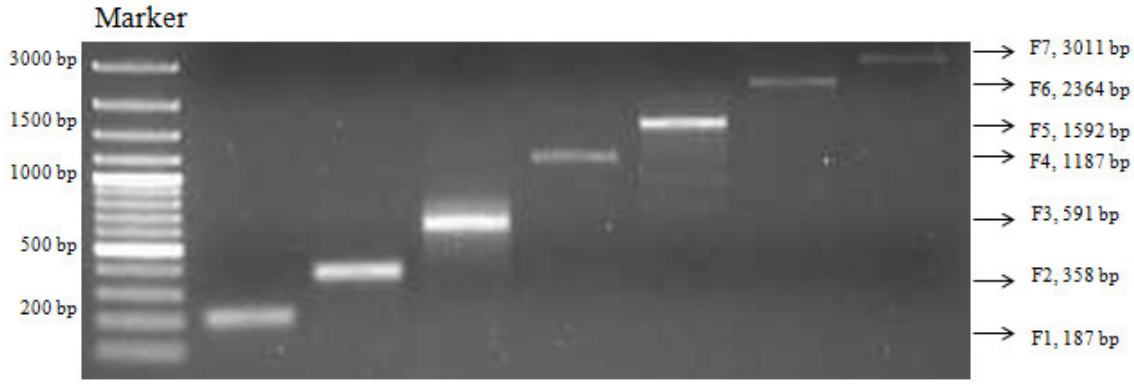

Figure 3.2. Seven different fragments amplified with PCR

3.3. TA Cloning and Confirmation with Restriction Enzyme Digestion

All purified seven DNA fragments were cloned with TOPO TA Cloning Kit (Invitrogen, K4520-01) and confirmed with EcoRI digestion. Each fragment was screened with four colonies as examplified with F6 fragment shown in Figure 3.3.

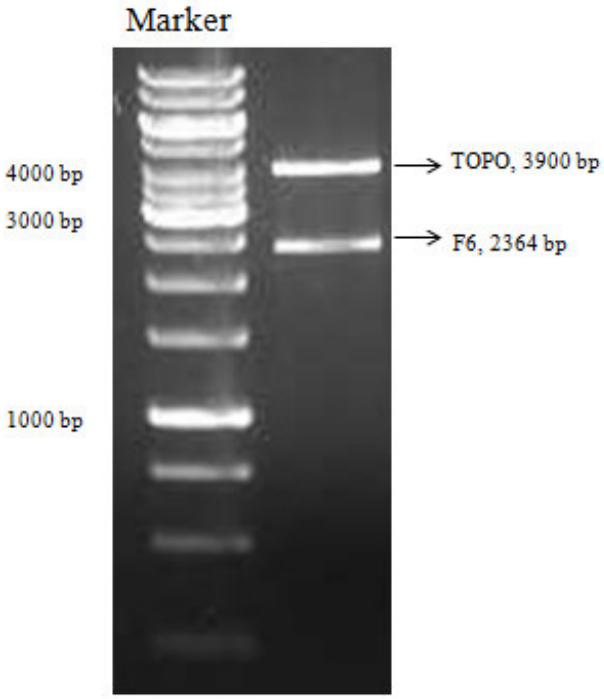

Figure 3.3. Screening of F6 TA cloning

3.4. Sequence Analysis of the Cloned Fragments and BLAST Search

The sequences obtained from Izmir Institute of Technology, Biotechnology and Bioengineering Research and Application Center were analysed using BLAST database and we confirmed that all the fragments matched with Neu4 gene in human genome and there is no Taq DNA Polymerase error. A typical BLAST output is shown in Figure 3.4.

3.5. Propagation and Confirmation of Firefly and Renilla Plasmids

Confirmation of pGL4.12 with PstI and AccI digestion and confirmation of pGL 4.74 with AlwNI and BalI digestion were shown in Figure 3.5. For pGL 4.12; PstI digestion produced 3057 bp and 1364 bp sequences, AccI digestion produced 2741 bp and 1680 bp sequences. For pGL 4.74; AlwNI digestion produced 2309 bp and 1928 bp sequences, BalI digestion produced 3278 bp and 959 bp sequences as confirmed on 1% agarose gel electrophoresis shown in Figure 3.5.

>ref|NT_005416.13| Homo sapiens chromosome 2 genomic contig, GRCh37 reference primary assembly
Length=2380241

Features in this part of subject sequence:
sialidase 4

Score = 346 bits (187), Expect = 4e-92
Identities = 187/187 (100%), Gaps = 0/187 (0%)
Strand=Plus/Plus

```
Query  70       CTCCTGGGTGCCCATCTGCACCCCTGGCCCTGCTCTGGGCTTCAGTGGAGCCGTGCCACC  129
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1943222  CTCCTGGGTGCCCATCTGCACCCCTGGCCCTGCTCTGGGCTTCAGTGGAGCCGTGCCACC  1943281

Query  130      GTGGGAGTGTCCAGGGTCCTGACGTAGCCCTGAGACGTGGCCAGCTCCCTGGCCTGCTGC  189
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1943282  GTGGGAGTGTCCAGGGTCCTGACGTAGCCCTGAGACGTGGCCAGCTCCCTGGCCTGCTGC  1943341

Query  190      CCGCACAGTGGGCCCTTGTCTCTGCTCTGGGTCAGGCGAGGCAGCCACCCATGATGAGCT  249
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1943342  CCGCACAGTGGGCCCTTGTCTCTGCTCTGGGTCAGGCGAGGCAGCCACCCATGATGAGCT  1943401

Query  250      CTGCAGC  256
                |||||||
Sbjct  1943402  CTGCAGC  1943408
```

Figure 3.4. BLAST output of F1 sequence

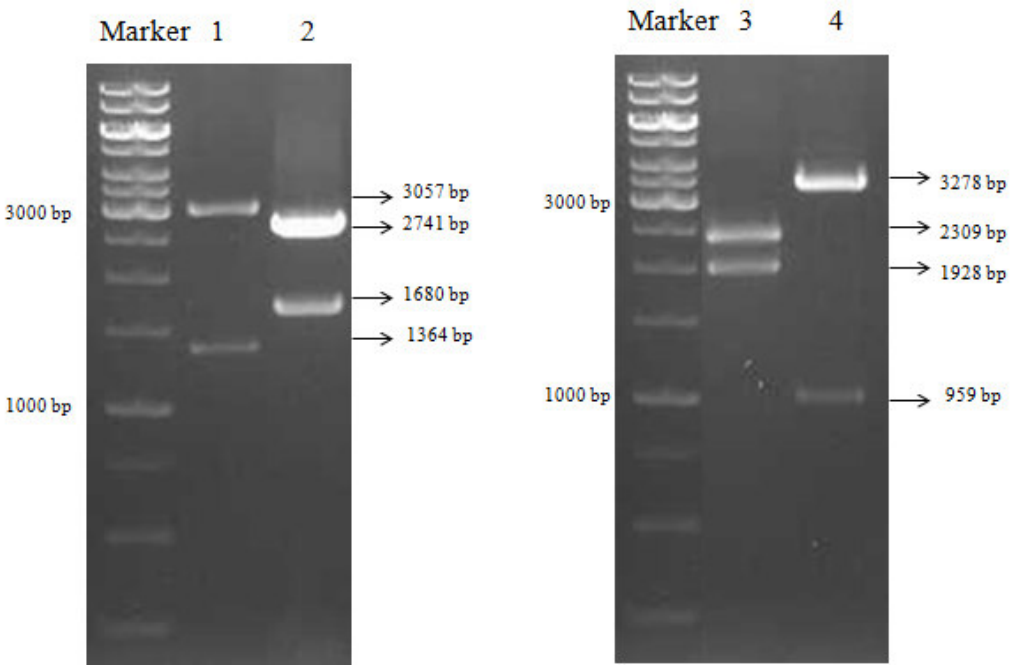

Figure 3.5. Confirmation of pGL 4.12 and pGL 4.74. 1, 2 are PstI and AccI digestions of pGL 4.12, respectively. 3, 4 are AlwNI and BalI digestions of pGL 4.74, respectively.

3.6. Cloning of the Fragments into pGL 4.12 Firefly Vector

pGL 4.12 was seperately digested with KpnI and NheI enzymes in contrast to all fragments were digested from pCR2.1 TOPO vector at the same time. Fragments were ligated with pGL 4.12 and propagated with bacterial transformation. Cloned expression vectors were confirmed with double digestion with KpnI and NheI enzymes as shown in Figure 3.6.

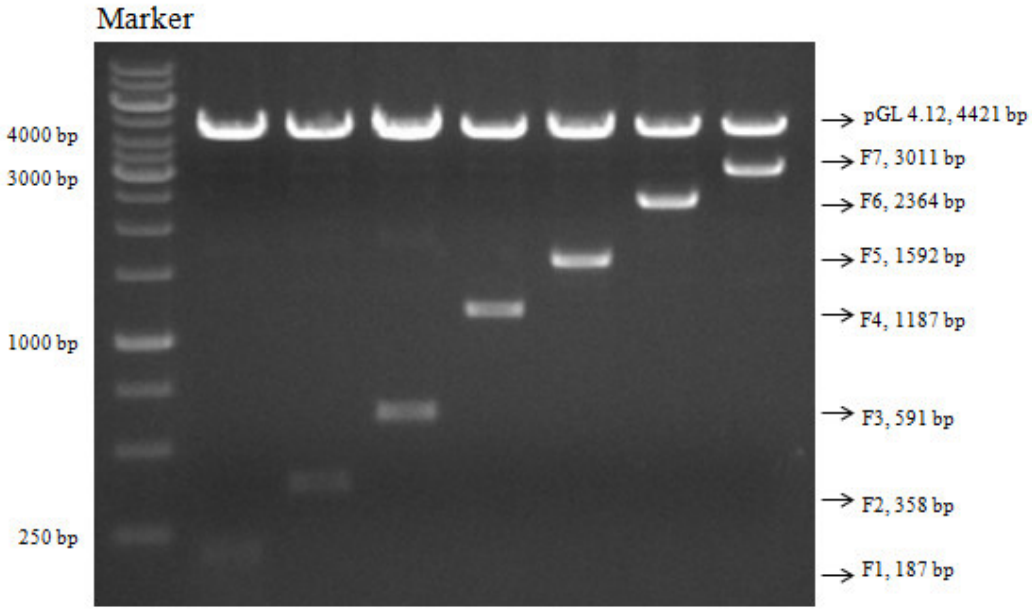

Figure 3.6. Restriction enzyme confirmation of seven different fragments cloned into pGL 4.12 vector

3.7. Optimization of Dual Luciferase Reporter (DLR) Assay System

DLR assay was optimized with ratios of 10:1 (pGL 4.12 : pGL 4.74) , respectively and 30:1(pGL 4.12 : pGL 4.74) , respectively for both 24 hours incubation and 48 hours incubation times in duplicate. Luminescence obtained from samples are listed in Table 3.1 below.

Table 3.1. Optimization of DLR assay

Ratios	Vectors	24 hours		48 hours	
		1	2	1	2
Blank	Firefly	3316	857	0	4547
	Renilla	25400	592100	10850	22870
10:1	Firefly	5565	8427	4021	4671
	Renilla	96510	99700	268600	170600
30:1	Firefly	11450	11700	2286	4405
	Renilla	32920	36240	25530	27360

Averages of blank firefly and renilla luminescence for 24 hours incubation were calculated seperately and the firefly average was subtracted from firefly values of 10:1 ratio and 30:1 ratio, the renilla average was subtracted from renilla values of 10:1 ratio and 30:1 ratio as well. Same calculations were performed for 48 hours incubation. Subtracted firefly values were divided with subtracted renilla values and averages of these ratios were calculated as shown in Table 3.2.

Table 3.2. Averages of luminescences

Ratio		24 hours			48 hours		
		1	2	Average	1	2	Average
10:1	F/R	0,048	0,085	0,067	0	0,0008	0,0004
30:1	F/R	1,245	0,886	1,065	0	0	0

According to optimization assays, 30:1 ratio of pGL 4.12 : pGL 4.74 incubated for 24 hours is the most efficient transfection condition for measuring luminescence of test samples.

3.8. Luminometrical Measurement of Seven Fragments

HeLa cells were transfected with each construct on the same day and additionally three independents assays (n=3) were performed different days as triplicates. All independent assays included blank, negative control and test samples. Luminescence obtained from samples are listed in Table 3.3 – 3.5 – 3.7 below. Averages of blank firefly and blank renilla luminescence values were subtracted from both negative controls and test samples as performed in optimization assay's calculations. After subtraction, fold activities were calculated with the Equation 3.1 below using renilla luminescences to normalize firefly values. Table 3.3 – 3.4, Figure 3.7 below show fold activities for assay 1. Table 3.5 – 3.6, Figure 3.8 below show fold activities for assay 2 and Table 3.7 – 3.8, Figure 3.9 below show fold activities for assay 3.

$$\Delta \text{ Fold Activity} = \frac{\text{Average (Firefly/Renilla) from Sample}}{\text{Average (Firefly/Renilla) from pGL4-Basic}} \qquad (3.1)$$

3.8.1. Assay 1

Table 3.3. Measured luminescence values (n=1)

n=1	Light Units	Blank	Negative Control	F1	F2	F3	F4	F5	F6	F7
1	Firefly	342.4	3792	32430	60200	69610	78030	34480	57380	39900
	Renilla	2343	27790	63990	68380	73960	79550	76840	87020	71950
2	Firefly	0	7147	32440	95310	85210	83130	38660	64310	62830
	Renilla	2370	57540	72260	94990	80200	100200	88170	103100	91300
3	Firefly	0	7933	58520	81540	95670	98250	45220	62750	63380
	Renilla	2362	60540	118600	74080	89930	111500	112800	115500	65440

Table 3.4. Calculated fold activities of assay (n=1)

n=1	Light Units	Negative Control	F1	F2	F3	F4	F5	F6	F7
1	F/R	0,1356	0,5206	0,9066	0,9674	1,0064	0,4583	0,6737	0,5684
2	F/R	1.1233	0,4591	1,0252	1,0902	0,8461	0,4465	0,6349	0,7025
3	F/R	0,1304	0,5004	1,1321	1,0885	0,8970	0,4063	0,5515	0,9993
Average F/R		0,4631	0,4933	1,0213	1,0487	0,9165	0,4370	0,6200	0,7567
Δ Fold Activity			1,0652	2,2053	2,2645	1,9790	0,9436	1,3388	1,3720

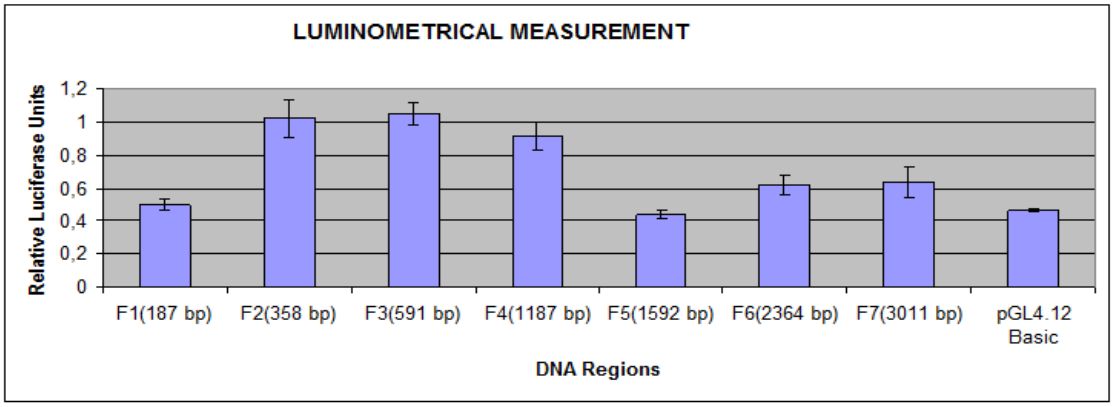

Figure 3.7. Graphic representing the fold activities of assay (n=1)

3.8.2. Assay 2

Table 3.5 Measured luminescence values (n=2)

n=2	Light Units	Blank	Negative Control	F1	F2	F3	F4	F5	F6	F7
1	Firefly	2496	9631	31770	26030	22560	32630	17200	30430	3645
	Renilla	4674	102200	77090	72450	44810	107900	123300	160100	9478
2	Firefly	1930	7289	28830	38400	46390	33830	14870	33900	11000
	Renilla	3618	77830	63290	97440	85330	100300	84200	147700	40740
3	Firefly	1912	10190	27220	63000	25970	15500	27950	53380	3144
	Renilla	3464	86670	45500	106900	41810	45120	102300	147000	11300

Table 3.6 Calculated fold activities of assay (n=2)

n=2	Light Units	Negative Control	F1	F2	F3	F4	F5	F6	F7
1	F/R	0,0764	0,4053	0,3489	0,5000	0,2934	0,1263	0,1813	0,2756
2	F/R	0,0700	0,4500	0,3880	0,5438	0,3290	0,1589	0,2210	0,2413
3	F/R	0,0976	0,6038	0,5912	0,6296	0,3249	0,2626	0,3583	0,1397
Average F/R		0,0813	0,4863	0,4427	0,5578	0,3157	0,1826	0,2435	0,2188
Δ Fold Activity			5,9815	5,4452	6,8610	3,8831	2,2460	3,1180	2,6912

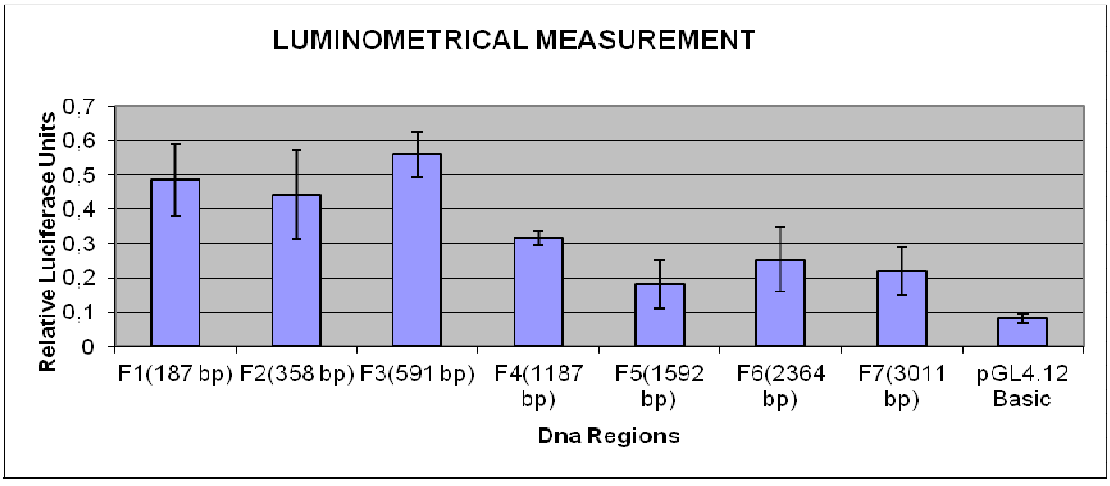

Figure 3.8. Graphic representing the fold activities of assay (n=2)

3.8.3. Assay 3

Table 3.7. Measured luminescence values (n=3)

n=3	Light Units	Blank	Negative Control	F1	F2	F3	F4	F5	F6	F7
1	Firefly	6291	20510	71350	89290	94840	64890	30830	53550	30040
	Renilla	14270	298600	160900	375700	260600	189500	292600	323000	293000
2	Firefly	4741	16190	55170	117600	99490	72820	35100	73100	53290
	Renilla	12190	281400	138700	402000	280600	214100	296400	333300	343200
3	Firefly	4114	20110	63960	117400	75520	88060	33780	98770	58720
	Renilla	9630	242800	88820	239100	145600	166800	200000	290300	304300

Table 3.8. Calculated fold activities of assay (n=3)

n=2	Light Units	Negative Control	F1	F2	F3	F4	F5	F6	F7
1	F/R	0,0539	0,4453	0,2316	0,3612	0,3371	0,0918	0,1559	0,0889
2	F/R	0,0413	0,3956	0,2886	0,3516	0,3353	0,1056	0,2118	0,1456
3	F/R	0,0652	0,7671	0,4947	0,5276	0,5363	0,1528	0,3368	0,1836
Average F/R		0,0534	0,5360	0,3383	0,4134	0,4029	0,1167	0,2368	0,1393
Δ Fold Activity			10,0374	6,3352	7,7415	7,5449	2,1853	4,4344	2,6086

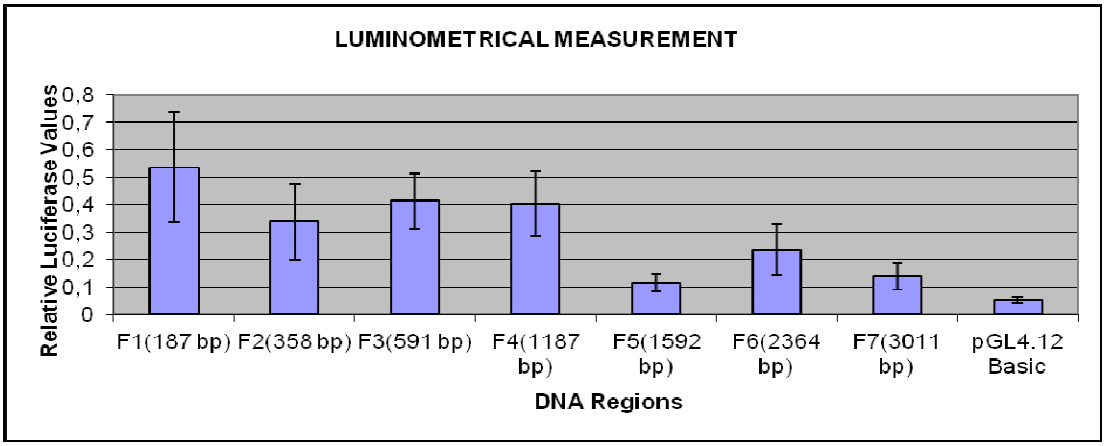

Figure 3.9 Graphic representing the fold activities of assay (n=3)

3.8.4. Fold Activities of All Measurements

All calculated fold changes were averaged together and standard deviations of these changes were calculated in MS Excel as shown in Table 3.9 and Figure 3.10.

Table 3.9. Average of all calculated assays and standard deviations

n	Light Units	Negative Control	F1	F2	F3	F4	F5	F6	F7
n=1	F/R	0,4631	1,0652	2,2053	2,2645	1,9790	0,9436	1,3388	1,3720
n=2	F/R	0,0813	5,9815	5,4452	6,8610	3,8831	2,2460	3,1180	2,6912
n=3	F/R	0,0534	10,0374	6,3352	7,7415	7,5449	2,1853	4,4344	2,6086
Average F/R		0,1992	5,6922	4,6601	5,6218	4,4673	1,7898	2,9489	2,2210
Average F/R Standard deviation		0,2289	4,4929	2,1735	2,9411	2,8288	0,7350	1,5535	0.7389
Δ Average F/R Fold Activity			19,6282	9,4954	12,8488	12,3582	3,2110	6,7868	3,2280

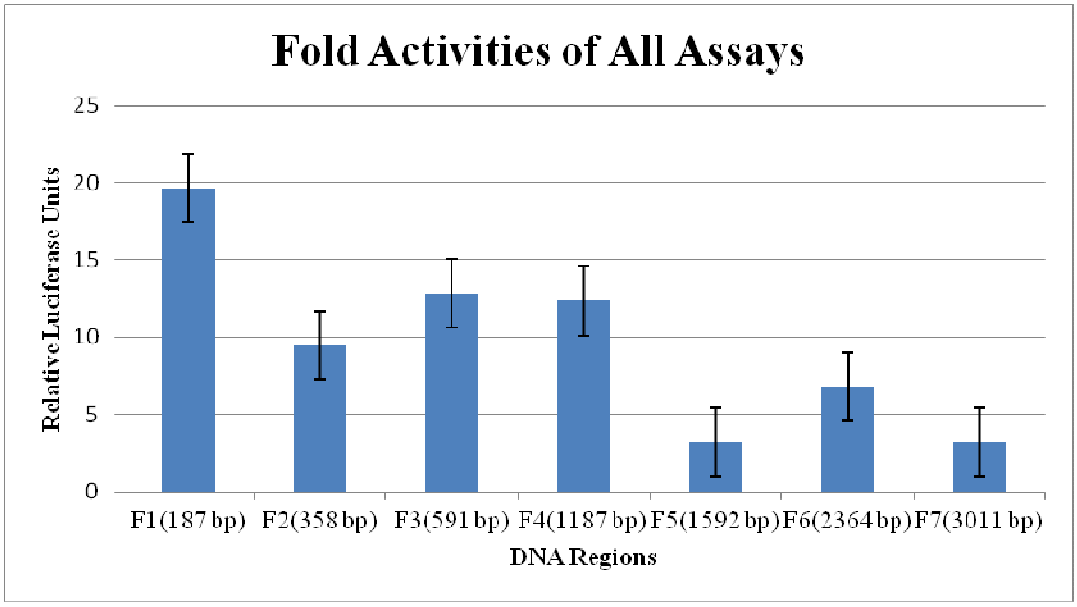

Figure 3.10. Graphic representing average of all assays with standard deviations

3.9. Electrophoretic Mobility Shift Assay (EMSA)

EMSA images representing free biotinilated DNA and DNA:protein complex (Shift) formation with 187 bp, 358 bp and 591 bp upstream regions are shown in Figure 3.11 – 3.12- 3.13 below.

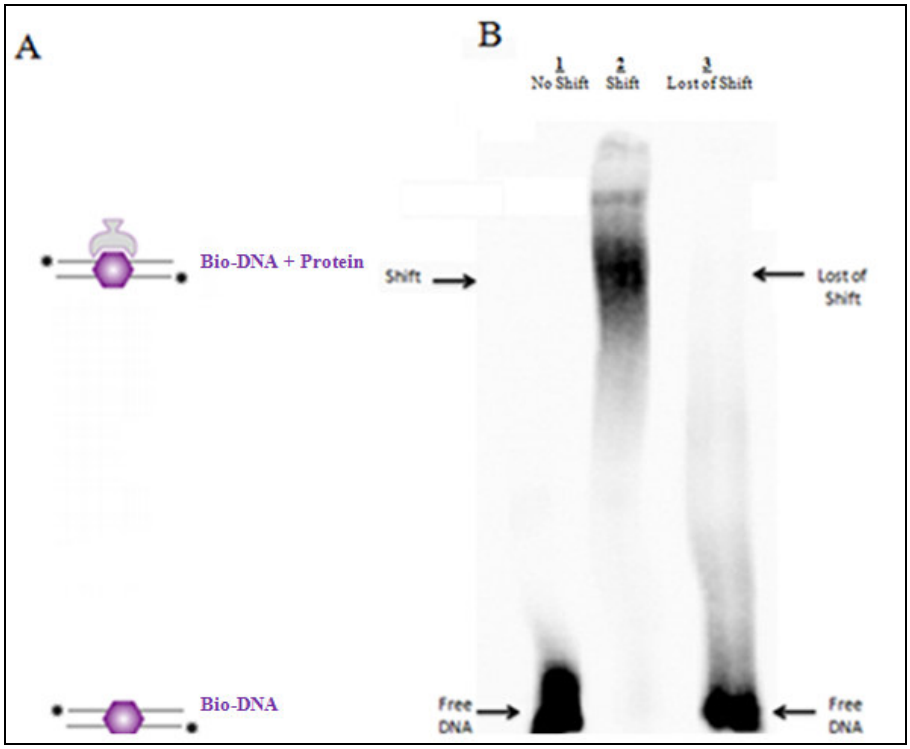

Figure 3.11. A, schematic presentation of a typical EMSA components. B, Image showing EMSA result of 187 bp upstream region (F1). 1, biotinilated free DNA; 2, DNA:protein complex (Shift), 3, competition assay proving specific binding of transcription factors to F1 fragment releasing biotinilated DNA free.

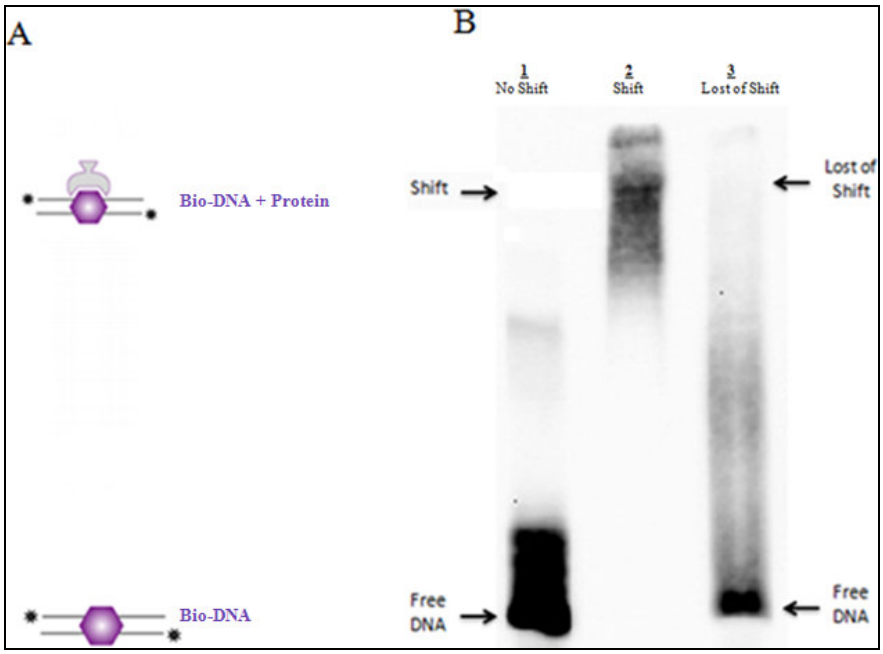

Figure 3.12. A, schematic presentation of a typical EMSA components. B, Image showing EMSA result of 358 bp upstream region (F2). 1, biotinilated free DNA; 2, DNA:protein complex (Shift), 3, competition assay providing specific binding of transcription factors to F2 fragment releasing biotinilated DNA free.

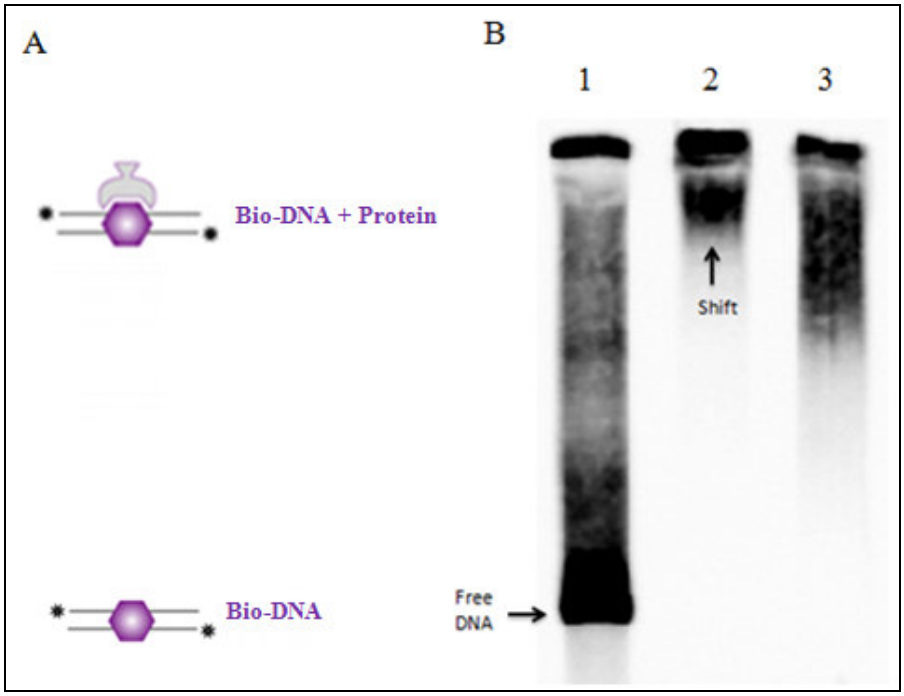

Figure 3.13. A, schematic presentation of a typical EMSA components. B, Image showing EMSA result of 591 bp upstream region (F3). 1, biotinilated free DNA; 2, DNA:protein complex (Shift), 3, competition assay proving specific binding of transcription factors to F3 fragment releasing biotinilated DNA free.

3.10. Supershift Assay with c-myc Antibody

EMSA image representing DNA:protein complex (Shift) formation and DNA:Protein:c-myc complex formation (Supershift) with 187 bp upstream region are shown in Figure 3.14.

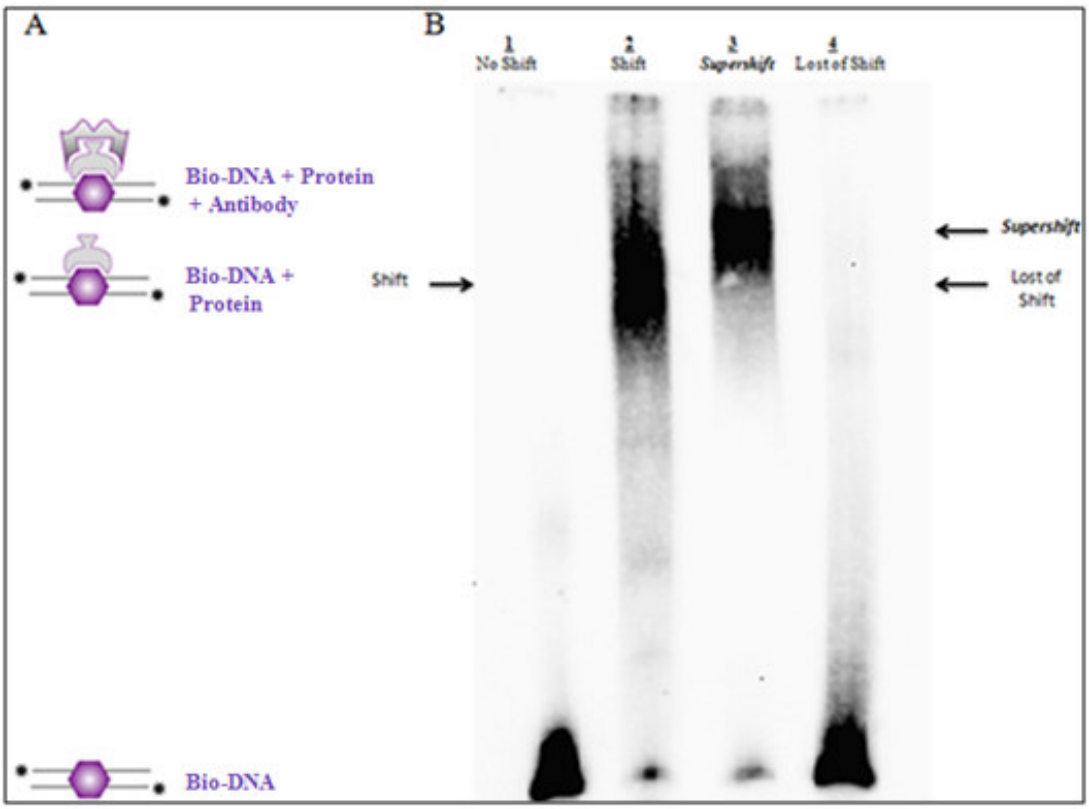

Figure 3.14. A, schematic presentation of a typical EMSA components. B, Image showing supershift result of 187 bp upstream region (F1) with c-myc antibody. 1, biotinilated free DNA; 2, DNA:protein complex (Shift), 3, supershift formation with c-myc antibody; 4, competition assay proving specific binding of transcription factors to F1 fragment releasing biotinilated DNA free.

CHAPTER 4

DISCUSSION

Neuraminidases (sialidases) show a wide distribution from viruses to vertebrates in nature. They remove sialic acid residues from glycoconjugates (Saito and Yu 1995). In human, four types of neuraminidases have been identified and cloned (Bonten 2000). Lysosomal/mitochondrial neuraminidase 4 (Neu4) is one of them and it has activity against sialylated glycoproteins, oligosaccharides, glycolipids in vitro (Seyrantepe et al. 2004; Monti et al. 2004). Neu4 deficient mice showed an increase in G_{D1a} ganglioside levels and a decrease in G_{M1} ganglioside levels in brain indicating activity of Neu4 against gangliosides. In addition lung and spleen of Neu4 deficient mice revealed vacuolization and lysosomal storage (Seyrantepe et al. 2008).

In this study, our aim is to perform molecular analysis of the 5' upstream regulatory region of human Neu4 gene. Although biochemical properties of human sialidase Neu4 enzyme was reported, Neu4 promoter region has not been studied yet. The data we obtained in this study provided an important clue about the molecular mechanism regulating human Neu4 gene expression. Here, we focused on 3000 bp upstream region of human Neu4 as a candidate promoter region. Bioinformatic analysis accomplished using TESS (Transcription Element Search System) tool revealed several putative transcription factor binding sites like c-myc, SP-1, YY1, AP-1 etc. in that region. TATA Box Hunting research tool using Genomatix revealed the absence of TATA box. In addition, the analysis of GC boxes using MBCF (Molecular Biology Core Facilities). Oligo Calculator revealed that 3000 bp upstream of human Neu4 has 61% GC content indicating potential transcription binding sites. We amplified and cloned seven different DNA fragments and sequenced them to confirm Taq Polymerase errors. Dual luciferase reporter system was used due to its experimental accuracy and high sensitivity as it can report femtograms of luciferase. Amplified DNA fragments were subcloned into pGL 4.12 vector. Optimization assays showed that parameters including the ratio of 30:1 (pGL 4.12:pGL 4.74, respectively) and 24 hours incubation time are optimum for Dual Luciferase Reporter System. All reporter assays were repeated three times. The data obtained using Dual Luciferase Reporter System showed

that 187 base pair upstream region (F1 fragment) has minimal transcriptional regulatory activity for human Neu4 gene with its highest luminescence among the others. We found that fold activity of luminescence gradually decreased between 187 bp and 1187 bp region. On the other hand, 358 upstream region showed a significant decrease in fold activities due to potential elements probably responsible for down-regulation of Neu4 gene. In addition, significantly decreased fold activities of luminescence were detected in 1592 bp, 2364 bp, 3011 bp upstream regions due to the effect of potential silencer elements. For instance, 187 bp region (F1 fragment) that has dominant promoter activity showed six times higher fold activity than 1592 bp region (F5 fragment). EMSA revealed that 187 bp, 358 bp, 591 bp upstream regions recruits some transcription factors as seen in 'Shift' formation with incubation of biotinilated DNA with nuclear proteins. Addition of unlabelled 187 bp, 358 bp, 591 bp regions into binding reactions proved the specificity of binding of transcription factors to candidate promoter region. EMSA also confirmed another supporting evidence for regulatory role of 187 bp upstream region (F1 fragment) as we determined formation of 'specific shift' at protein level besides its highest luminescence fold activity. In particular, we showed the binding of c-myc to our minimal promoter region by supershift assay. The highest luminescence obtained from 187 bp upstream region (F1 fragment) of human Neu4 gene may be a result of predicted transcription factor binding sites for c-myc which is also responsible for transcription regulation of many genes involved cellular events such as cell proliferation and differentiation.

CHAPTER 5

CONCLUSION

In this study we report the molecular characterization of promoter region of human Neu4 gene for the first time. Our lucifarese reporter assays demostrated 187 bp TATA-less promoter is required for minimal activity. Upstream regions from 187 bp showed a decrease in activity. Our EMSA results supported the specific binding of transcription factors such as c-myc at protein level to the same region.

In the future, the data we obtained can be used for discovering small molecules which may control human Neu4 gene expression. Selective high expression or silencing of Neu4 gene might be achieved using drugs or small molecules. High expression of Neu4 gene and increased level of Neu4 neuraminidase in tissues can clear accumulated G_{M2} ganglioside in lysosomes of Tay-Sachs patients due to beta-hexosaminidase A deficiency.

It has been shown that Neu4 is downregulated in human colon cancer cells and overexpression of Neu4 in cultured cells accelerates apoptosis and decreases invasiveness and motility. Research to study the upregulation of human Neu4 gene expression by selective binding of c-myc protein into the promoter region might be important to treat human colon cancer.

In addition, the importance of up and/or down regulation of Neu4 gene expression in degradation of biomolecules, cellular communication, cell growth and differentiation and cell death can be enlightened.

REFERENCES

Akita, H., Miyagi, T., Hata, K., & Kagayama, M. (1997). Immunohistochemical evidence for the existence of rat cytosolic sialidase in rat skeletal muscles. *Histochemistry and cell biology*, *107*(6), 495-503.

Berg. (2005). *Lysosomes,* edited by Paul Saftig. ©2005 Eurekah.com and Springer Science and Business Media.

Bigi, A., Morosi, L., Pozzi, C., Forcella, M., Tettamanti, G., Venerando, B., Monti, E., et al. (2010). Human sialidase NEU4 long and short are extrinsic proteins bound to outer mitochondrial membrane and the endoplasmic reticulum, respectively. *Glycobiology*, *20*(2), 148-57.

Blackwood, E. M., & Kadonaga, J. T. (1998). Going the distance: a current view of enhancer action. *Science (New York, N.Y.)*, *281*(5373), 60-3.

Bonten, E., van der Spoel, a, Fornerod, M., Grosveld, G., & d' Azzo, a. (1996). Characterization of human lysosomal neuraminidase defines the molecular basis of the metabolic storage disorder sialidosis. *Genes & Development*, *10*(24), 3156-3169.

Brett, E. M., Ellis, R. B., Haas, L., Ikonne, J. U., Lake, B. D., Patrick, A. D., & Stephens, R. (1973). Late onset GM2-gangliosidosis. Clinical, pathological, and biochemical studies on 8 patients. *Archives of disease in childhood*, *48*(10), 775-85.

Bulyk, M. L. (2003). Computational prediction of transcription-factor binding site locations. *Genome biology*, *5*(1), 201.

Caimi, L., Tettamanti, G., Berra, B., Omodeo Sale, F., Borrone, C., Gatti, R., Durand, P., et al. (1982). Mucolipidosis IV, a sialolipidosis due to ganglioside sialidase deficiency. *Journal of inherited metabolic disease*, *5*(4), 218-24.

Carey, M., Smale, S. Judy Cuddihy, Patricia Barker, Danny deBruin, Susan Schaefer (eds) *Transcriptional Regulation in Eukaryotes: Concepts, Strategies, and Techniques.* Cold Spring Harbor Laboratory Press, USA, 2001:3–20, 194–212.

Chen, H., & Chang, G. D. (2001). Simultaneous immunoblotting analysis with activity gel electrophoresis in a single polyacrylamide gel. *Electrophoresis*, *22*(10), 1894-9.

Cohen-Tannoudji, M., Marchand, P., Akli, S., Sheardown, S. A., Puech, J. P., Kress, C., Gressens, P., et al. (1995). Disruption of murine Hexa gene leads to enzymatic deficiency and to neuronal lysosomal storage, similar to that observed in Tay-Sachs disease. *Mammalian genome: official journal of the International Mammalian Genome Society*, *6*(12), 844-9.

de Wet, J. R., Wood, K. V., DeLuca, M., Helinski, D. R., & Subramani, S. (1987). Firefly luciferase gene: structure and expression in mammalian cells. *Molecular and cellular biology*, *7*(2), 725-37.

de Wet, J. R., Wood, K. V., Helinski, D. R., & DeLuca, M. (1985). Cloning of firefly luciferase cDNA and the expression of active luciferase in Escherichia coli. *Proceedings of the National Academy of Sciences of the United States of America*, *82*(23), 7870-3.

Durand, P., Gatti, R., Cavalieri, S., Borrone, C., Tondeur, M., Michalski, J. C., & Strecker, G. (1977). Sialidosis (mucolipidosis I). *Helvetica paediatrica acta*, *32*(4-5), 391-400.

Epstein, D. J. (2009). Cis-regulatory mutations in human disease. *Briefings in functional genomics & proteomics*, *8*(4), 310-6.

Fan, F., & Wood, K. V. (2007). Bioluminescent assays for high-throughput screening. *Assay and drug development technologies*, *5*(1), 127-36.

Fanzani, A., Giuliani, R., Colombo, F., Zizioli, D., Presta, M., Preti, A., & Marchesini, S. (2003). Overexpression of cytosolic sialidase Neu2 induces myoblast differentiation in C2C12 cells. *FEBS letters*, *547*(1-3), 183-8.

Federico, A. (1987). GM2 gangliosidosis with a motor neuron disease phenotype: clinical heterogeneity of hexosaminidase deficiency disease. *Advances in experimental medicine and biology*, *209*, 19-23.

Forwood, J. K., & Jans, D. A. (2006). Quantitative analysis of DNA-protein interactions using double-labeled native gel electrophoresis and fluorescence-based imaging. *Electrophoresis*, *27*(16), 3166-70.

Fried, M. G. (n.d.). Measurement of protein-DNA interaction parameters by electrophoresis mobility shift assay. *Electrophoresis*, *10*(5-6), 366-76.

Fried, M. G., & Crothers, D. M. (1984). Kinetics and mechanism in the reaction of gene regulatory proteins with DNA. *Journal of molecular biology*, *172*(3), 263-82.

Fried, M., & Crothers, D. M. (1981). Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. *Nucleic acids research*, *9*(23), 6505-25.

Garner, M. M., & Revzin, A. (1981). A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the Escherichia coli lactose operon regulatory system. *Nucleic acids research*, *9*(13), 3047-60.

Gaszner, M., & Felsenfeld, G. (2006). Insulators: exploiting transcriptional and epigenetic mechanisms. *Nature reviews. Genetics*, *7*(9), 703-13.

Gomez-Lira, M., Sangalli, A., Mottes, M., Perusi, C., Pignatti, P. F., Rizzuto, N., & Salviati, A. (1995). A common beta hexosaminidase gene mutation in adult Sandhoff disease patients. *Human genetics*, *96*(4), 417-22.

Granger-Schnarr, M., Lloubes, R., de Murcia, G., & Schnarr, M. (1988). Specific protein-DNA complexes: immunodetection of the protein component after gel electrophoresis and Western blotting. *Analytical biochemistry*, *174*(1), 235-8.

Gravel, R. A., Clarke, J. T. R., Kaback, M. M., Mahuran, D., Sandhoff, K., Suzuki K. C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), *The Metabolic and Molecular Bases of Inherited Disease,* Vol. 2, 7 edn., McGraw-Hill, New York, 1995, pp. 2839-2879.

Grosso, S., Farnetani, M. A., Berardi, R., Margollicci, M., Galluzzi, P., Vivarelli, R., Morgese, G., et al. (2003). GM2 gangliosidosis variant B1 neuroradiological findings. *Journal of neurology*, *250*(1), 17-21.

Harding, A. E., Young, E. P., & Schon, F. (1987). Adult onset supranuclear ophthalmoplegia, cerebellar ataxia, and neurogenic proximal muscle weakness in a brother and sister: another hexosaminidase A deficiency syndrome. *Journal of neurology, neurosurgery, and psychiatry*, *50*(6), 687-90.

Hart, C. M., & Laemmli, U. K. (1998). Facilitation of chromatin dynamics by SARs. *Current opinion in genetics & development*, *8*(5), 519-25.

Heim, R., Cubitt, A. B., & Tsien, R. Y. (1995). Improved green fluorescence. *Nature*, *373*(6516), 663-4.

Hendrickson, W. (1985). *Biotechniques* **3**, 346-354.

James, A. L., Perry, J. D., Ford, M., Armstrong, L., & Gould, F. K. (1996). Evaluation of cyclohexenoesculetin-beta-D-galactoside and 8-hydroxyquinoline-beta-D-galactoside as substrates for the detection of beta-galactosidase. *Applied and environmental microbiology*, *62*(10), 3868-70.

Johnson, W. G., Cohen, C. S., Miranda, A. F., Waran, S. P., & Chutorian, A. M. (1980). Alpha-locus hexosaminidase genetic compound with juvenile gangliosidosis phenotype: clinical, genetic, and biochemical studies. *American journal of human genetics*, *32*(4), 508-18.

Kang, J., Lee, M. S., & Gorenstein, D. G. (2005). Quantitative analysis of chemiluminescence signals using a cooled charge-coupled device camera. *Analytical biochemistry*, *345*(1), 66-71.

Kaplan, C. D., Laprade, L., & Winston, F. (2003). Transcription elongation factors repress transcription initiation from cryptic sites. *Science (New York, N.Y.)*, *301*(5636), 1096-9.

Katranidis, A., Atta, D., Schlesinger, R., Nierhaus, K. H., Choli-Papadopoulou, T., Gregor, I., Gerrits, M., et al. (2009). Fast biosynthesis of GFP molecules: a single-molecule fluorescence study. *Angewandte Chemie (International ed. in English)*, *48*(10), 1758-61.

Kelly, T. E., & Graetz, G. (1977). Isolated acid neuraminidase deficiency: a distinct lysosomal storage disease. *American journal of medical genetics*, *1*(1), 31-46.

Khoury, G., & Gruss, P. (1983). Enhancer elements. *Cell*, *33*(2), 313-4.

Kolodny, E. H., (1966). The GM2 gangliosidoses. In Rosenberg RN, Prusiner SB, et al. (Eds.), *The Molecular and Genetic Basis of Neurological Disease*. 2nd ed. Boston: Butterworth Heinemann.

Kopitz, J., von Reitzenstein, C., Burchert, M., Cantz, M., & Gabius, H. J. (1998). Galectin-1 is a major receptor for ganglioside GM1, a product of the growth-controlling activity of a cell surface ganglioside sialidase, on human neuroblastoma cells in culture. *The Journal of biological chemistry*, *273*(18), 11205-11.

Kristie, T. M., & Roizman, B. (1986). Alpha 4, the major regulatory protein of herpes simplex virus type 1, is stably and specifically associated with promoter-regulatory domains of alpha genes and of selected other viral genes. *Proceedings of the National Academy of Sciences of the United States of America*, *83*(10), 3218-22.

Kummerfeld, S. K., & Teichmann, S. A. (2006). DBD: a transcription factor prediction database. *Nucleic acids research*, *34*(Database issue), D74-81.

Lane, D., Prentki, P., & Chandler, M. (1992). Use of gel retardation to analyze protein-nucleic acid interactions. *Microbiological reviews*, *56*(4), 509-28.

Lee, G. R., Fields, P. E., Griffin, T. J., & Flavell, R. A. (2003). Regulation of the Th2 cytokine locus by a locus control region. *Immunity*, *19*(1), 145-53.

Lee, M. P., Howcroft, K., Kotekar, A., Yang, H. H., Buetow, K. H., & Singer, D. S. (2005). ATG deserts define a novel core promoter subclass. *Genome research*, *15*(9), 1189-97.

Li, Q., Peterson, K. R., Fang, X., & Stamatoyannopoulos, G. (2002). Locus control regions. *Blood*, *100*(9), 3077-86.

Li, Y., Jiang, Z., Chen, H., & Ma, W.-J. (2004). A modified quantitative EMSA and its application in the study of RNA--protein interactions. *Journal of biochemical and biophysical methods*, *60*(2), 85-96.

Liang, F., Seyrantepe, V., Landry, K., Ahmad, R., Ahmad, A., Stamatos, N. M., & Pshezhetsky, A. V. (2006). Monocyte differentiation up-regulates the expression of the lysosomal sialidase, Neu1, and triggers its targeting to the plasma membrane via major histocompatibility complex class II-positive compartments. *The Journal of biological chemistry*, *281*(37), 27526-38.

Lim, C. Y., Santoso, B., Boulay, T., Dong, E., Ohler, U., & Kadonaga, J. T. (2004). The MTE, a new core promoter element for transcription by RNA polymerase II. *Genes & development*, *18*(13), 1606-17.

Lukong, K. E., Landry, K., Elsliger, M. A., Chang, Y., Lefrancois, S., Morales, C. R., & Pshezhetsky, A. V. (2001). Mutations in sialidosis impair sialidase binding to the lysosomal multienzyme complex. *The Journal of biological chemistry*, *276*(20), 17286-90.

Lukong, K. E., Seyrantepe, V., Landry, K., Trudel, S., Ahmad, A., Gahl, W. A., Lefrancois, S., et al. (2001). Intracellular distribution of lysosomal sialidase is controlled by the internalization signal in its cytoplasmic tail. *The Journal of biological chemistry*, *276*(49), 46172-81.

Madan Babu, M., & Teichmann, S. A. (2003). Functional determinants of transcription factors in Escherichia coli: protein families and binding sites. *Trends in genetics: TIG*, *19*(2), 75-9.

Mark, B. L., Mahuran, D. J., Cherney, M. M., Zhao, D., Knapp, S., & James, M. N. G. (2003). Crystal structure of human beta-hexosaminidase B: understanding the molecular basis of Sandhoff and Tay-Sachs disease. *Journal of molecular biology*, *327*(5), 1093-109.

Maston, G. a, Evans, S. K., & Green, M. R. (2006). Transcriptional regulatory elements in the human genome. *Annual review of genomics and human genetics*, *7*, 29-59.

Meier, E. M., Schwarzmann, G., Fürst, W., & Sandhoff, K. (1991). The human GM2 activator protein. A substrate specific cofactor of beta-hexosaminidase A. *The Journal of biological chemistry*, *266*(3), 1879-87.

Milner, C. M., Smith, S. V., Carrillo, M. B., Taylor, G. L., Hollinshead, M., & Campbell, R. D. (1997). Identification of a sialidase encoded in the human major histocompatibility complex. *The Journal of biological chemistry*, *272*(7), 4549-58.

Mitchell, P. J., & Tjian, R. (1989). Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science (New York, N.Y.)*, *245*(4916), 371-8.

Miyagi, T. (2008). Review Aberrant expression of sialidase and cancer progression. *Cancer*, *84*(10), 407-418.

Monti, E., Bassi, M. T., Bresciani, R., Civini, S., Croci, G. L., Papini, N., Riboni, M., et al. (2004). Molecular cloning and characterization of NEU4, the fourth member of the human sialidase gene family. *Genomics*, *83*(3), 445-53.

Moriwaki, S., Takashima, S., Yoshida, H., Kawano, N., & Goto, M. (1977). Histological observation of the brain of Tay-Sachs disease with seizure and chronic DPH intoxication--report of an autopsy case. *Acta pathologica japonica*, *27*(3), 387-407.

National Library of Medicine. http://www.nlm.nih.gov (Accessed December 5, 2011)

Nardocci, N., Bertagnolio, B., Rumi, V., & Angelini, L. (1992). Progressive dystonia symptomatic of juvenile GM2 gangliosidosis. *Movement disorders: official journal of the Movement Disorder Society*, *7*(1), 64-7.

Narlikar, G. J., Fan, H.-Y., & Kingston, R. E. (2002). Cooperation between complexes that regulate chromatin structure and transcription. *Cell*, *108*(4), 475-87.

Narlikar, L., & Ovcharenko, I. (2009). Identifying regulatory elements in eukaryotic genomes. *Briefings in functional genomics & proteomics*, *8*(4), 215-30.

Neudorfer, O., Pastores, G. M., Zeng, B. J., Gianutsos, J., Zaroff, C. M., & Kolodny, E. H. (2005). Late-onset Tay-Sachs disease: phenotypic characterization and genotypic correlations in 21 affected patients. *Genetics in medicine: official journal of the American College of Medical Genetics*, *7*(2), 119-23.

Nilsson, C., Kågedal, K., Johansson, U., & Ollinger, K. (2003). Analysis of cytosolic and lysosomal pH in apoptotic cells by flow cytometry. *Methods in cell science: an official journal of the Society for In Vitro Biology*, *25*(3-4), 185-94.

O'Brien, J. S. (1978). The cherry red spot-myoclonus syndrome: a newly recognized inherited lysosomal storage disease due to acid neuraminidase deficiency. *Clinical genetics*, *14*(1), 55-60.

Ogbourne, S., & Antalis, T. M. (1998). Transcriptional control and the role of silencers in transcriptional regulation in eukaryotes. *The Biochemical journal*, *331 (Pt 1*, 1-14.

Ohno, K., & Suzuki, K. (1988). Mutation in GM2-gangliosidosis B1 variant. *Journal of neurochemistry*, *50*(1), 316-8.

Okada, S., & O'Brien, J. S. (1969). Tay-Sachs disease: generalized absence of a beta-D-N-acetylhexosaminidase component. *Science (New York, N.Y.)*, *165*(3894), 698-700.

OMIM. http://www.omim.org/entry/268800?search=sandhoff%20disease&highlight=sandhoff%20disease (Accessed December 5, 2011)

Phaneuf, D., Wakamatsu, N., Huang, J. Q., Borowski, A., Peterson, A. C., Fortunato, S. R., Ritter, G., et al. (1996). Dramatically different phenotypes in mouse models of human Tay-Sachs and Sandhoff diseases. *Human molecular genetics*, *5*(1), 1-14.

Pshezhetsky, A. V., & Ashmarina, M. (2001). Lysosomal multienzyme complex: biochemistry, genetics, and molecular pathophysiology. *Progress in nucleic acid research and molecular biology*, *69*, 81-114.

Pshezhetsky, A. V., Richard, C., Michaud, L., Igdoura, S., Wang, S., Elsliger, M. A., Qu, J., et al. (1997). Cloning, expression and chromosomal mapping of human lysosomal sialidase and characterization of mutations in sialidosis. *Nature genetics*, *15*(3), 316-20.

Rapin, I., Goldfischer, S., Katzman, R., Engel, J., & O'Brien, J. S. (1978). The cherry-red spot--myoclonus syndrome. *Annals of neurology*, *3*(3), 234-42.

Revzin, A. (1989). Gel electrophoresis assays for DNA-protein interactions. *BioTechniques*, *7*(4), 346-55.

Rye, H. S., Drees, B. L., Nelson, H. C., & Glazer, A. N. (1993). Stable fluorescent dye-DNA complexes in high sensitivity detection of protein-DNA interactions. Application to heat shock transcription factor. *The Journal of biological chemistry*, *268*(33), 25229-38.

Sachs, B. (1887). On arrested cerebral development, with special reference to its cortical pathology. *J Nerv Ment Disorders,* 14:541–553.

Saito, M., Yu, R.K., (1995). Biochemistry and function of sialidases. *Biology of the Sialic Acids, Rosenberg, editor. Plenum Press, New York.* 261-313.

Sandhoff, K., Andreae, U., & Jatzkewitz, H. (1968). Deficient hexozaminidase activity in an exceptional case of Tay-Sachs disease with additional storage of kidney globoside in visceral organs. *Life sciences*, *7*(6), 283-8.

Sango, K., Yamanaka, S., Hoffmann, A., Okuda, Y., Grinberg, A., Westphal, H., McDonald, M. P., et al. (1995). Mouse models of Tay-Sachs and Sandhoff diseases differ in neurologic phenotype and ganglioside metabolism. *Nature genetics*, *11*(2), 170-6.

Sato, K., & Miyagi, T. (1996). Involvement of an endogenous sialidase in skeletal muscle cell differentiation. *Biochemical and biophysical research communications*, *221*(3), 826-30.

Schneider-Jakob, H. R., & Cantz, M. (1991). Lysosomal and plasma membrane ganglioside GM3 sialidases of cultured human fibroblasts. Differentiation by detergents and inhibitors. *Biological chemistry Hoppe-Seyler*, *372*(6), 443-50.

Seyrantepe, V., Landry, K., Trudel, S., Hassan, J. a, Morales, C. R., & Pshezhetsky, A. V. (2004). Neu4, a novel human lysosomal lumen sialidase, confers normal phenotype to sialidosis and galactosialidosis cells. *The Journal of biological chemistry*, *279*(35), 37021-9.

Seyrantepe, V., Canuel, M., Carpentier, S., Landry, K., Durand, S., Liang, F., Zeng, J., et al. (2008). Mice deficient in Neu4 sialidase exhibit abnormal ganglioside catabolism and lysosomal storage. *Human molecular genetics*, *17*(11), 1556-68.

Seyrantepe, V., Lema, P., Caqueret, A., Dridi, L., Bel Hadj, S., Carpentier, S., Boucher, F., et al. (2010). Mice doubly-deficient in lysosomal hexosaminidase A and neuraminidase 4 show epileptic crises and rapid neuronal loss. *PLoS genetics*, *6*(9).

Smale, S. T., & Kadonaga, J. T. (2003). The RNA polymerase II core promoter. *Annual review of biochemistry*, *72*, 449-79.

Specola, N., Vanier, M. T., Goutières, F., Mikol, J., & Aicardi, J. (1990). The juvenile and chronic forms of GM2 gangliosidosis: clinical and enzymatic heterogeneity. *Neurology*, *40*(1), 145-50.

Stamatos, N. M., Liang, F., Nan, X., Landry, K., Cross, A. S., Wang, L.-X., & Pshezhetsky, A. V. (2005). Differential expression of endogenous sialidases of human monocytes during cellular differentiation into macrophages. *The FEBS journal*, *272*(10), 2545-56.

Steghens, J. P., Min, K. L., & Bernengo, J. C. (1998). Firefly luciferase has two nucleotide binding sites: effect of nucleoside monophosphate and CoA on the light-emission spectra. *The Biochemical journal*, *336 (Pt 1*, 109-13.

Suzuki, K., (1987). Enzymatic diagnosis of sphingolipidoses. Ginsbury V (ed) *Methods in enzymology, vol 138. Academic, New York,* pp 727–762.

Suzuki, K., (1994). Genetic disorders of lipid, glycoprotein, and mucopolysaccharide metabolism. *Basic Neurochemistry: Molecular, Cellular, and Medical Aspects*, *ed. GJ Siegel, BW Agranoff, RW Albers, PB Molinoff, pp. 793–812. New York: Raven. 5th ed*

Thomas, G. H. (2001). Disorders of glycoprotein Degradation: a-mannosidosis, b-mannosidosis, fucosidosis, and sialidosis. Scriver CR, Beaudet AL, Sly WS, Valle D, editors. *The metabolic and molecular bases of inherited disease, New York: McGraw-Hill. p* 3507–3534.

Triggs-Raine, B. L., Mules, E. H., Kaback, M. M., Lim-Steele, J. S., Dowling, C. E., Akerman, B. R., Natowicz, M. R., et al. (1992). A pseudodeficiency allele common in non-Jewish Tay-Sachs carriers: implications for carrier screening. *American journal of human genetics*, *51*(4), 793-801.

Turk, B., Turk, D., & Turk, V. (2000). Lysosomal cysteine proteases: more than scavengers. *Biochimica et biophysica acta*, *1477*(1-2), 98-111.

Uniprot. http://www.uniprot.org/uniprot/Q99519 (Accessed December 5, 2011)

Wada, T., Yoshikawa, Y., Tokuyama, S., Kuwabara, M., Akita, H., & Miyagi, T. (1999). Cloning, expression, and chromosomal mapping of a human ganglioside sialidase. *Biochemical and Biophysical Research Communications*, *261*(1), 21-27.

Wang, Y., Yamaguchi, K., Wada, T., Hata, K., Zhao, X., Fujimoto, T., & Miyagi, T. (2002). A close association of the ganglioside-specific sialidase Neu3 with caveolin in membrane microdomains. *The Journal of biological chemistry*, *277*(29), 26252-9.

Winter, R. M., Swallow, D. M., Baraitser, M., & Purkiss, P. (1980). Sialidosis type 2 (acid neuraminidase deficiency): clinical and biochemical features of a further case. *Clinical genetics*, *18*(3), 203-10.

Wood, K. V. (1995). Marker proteins for gene expression. *Current opinion in biotechnology*, *6*(1), 50-8.

Wood, K. V., de Wet, J. R., Dewji, N., & DeLuca, M. (1984). Synthesis of active firefly luciferase by in vitro translation of RNA obtained from adult lanterns. *Biochemical and biophysical research communications*, *124*(2), 592-6.