THE HUMAN LYSOSOMAL SIALIDASE PROMOTER

THE HUMAN LYSOSOMAL SIALIDASE PROMOTER: CHARACTERIZATION AND STIMULATION AS A POTENTIAL THERAPY FOR TAY-SACHS DISEASE

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Abstract

Tay-Sachs disease and its related disorders (GM2 gangliosidoses) are neurodegenerative diseases caused by the excessive accumulation of ganglioside GM2, an otherwise non-toxic plasma membrane component, in the lysosomes of cells of the central nervous system. The accumulation of ganglioside GM2 is the result of a defect in the gene encoding the α-subunit of β-hexosaminidase A (Hex A), an acid hydrolase responsible for the metabolism of gangloside GM2 in the lysosome. Though a debilitating disease in humans, Tay-Sachs model mice (*Hexa-/-*) escape symptoms by the action of lysosomal sialidase, which is expressed in mice at levels sufficient to metabolize ganglioside GM2 and effectively "bypass" Hex A deficiency. In an attempt to understand why a lysosomal sialidase-mediated bypass of Hex A deficiency is not observed in humans, we cloned ~ 2.9 kb of the human lysosomal sialdiase promoter and began characterization of the regulatory machinery that determines its activity. The transcription factor CDP (CCAAT-Displacement Protein) and truncations thereof were found to have a clear and consistent effect on promoter activity *in vitro*, with the truncation $CDP^{831\text{-}1505}$ resulting in a near 50-fold increase in activity. Adenovirus-mediated gene transfer of $CDP^{831\text{-}1505}$ into CRB/TSD cells, a human Tay-Sachs neuroglia cell line, resulted in elevated lysosomal sialidase activity and a decrease in ganglioside GM2 stores. These results suggest that the regulatory machinery responsible for lysosomal sialidase expression may be manipulated in such a way as to "activate" a sialidase-mediated bypass of Hex A deficiency in human Tay-Sachs cells. Thus, induction of lysosomal sialidase may have a potential therapeutic benefit in human Tay-Sachs disease and other Hex A-deficient GM2 gangliosidoses.

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List of Abbreviations

BAC	Bacterial Artificial Chromosome
bp	Base pair
cDNA	complimentary deoxyribonucleic acid
CDP	CCAAT-displacement protein
CHD	Cut homeodomain
CNS	Central Nervous System
CR1	Cut Repeat 1
CR2	Cut Repeat 2
CR3	Cut Repeat 3
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
ER	Endoplasmic Reticulum
ERT	Enzyme Replacement Therapy
GSL	Glycosphingolipid
Hex A	β-hexosaminidase A
Hex B	β-hexosamindase B
kb	kilo base pair
kDa	kilodalton
MAP	2-amino-2-methyl-1-propanol
MCB	membranous cytoplasmic body
mRNA	messenger ribonucleic acid

Mu-Nana	4-methylumbelliferyl-n-acetyl-α-D-neuramide
NADPH	nicotinamide adenine dinucleotide phosphate
NB-DNJ	*N*-butyldeoxynojirimycin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PPCA	protective protein/cathepsin A
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse-transcriptase polymerase chain reaction
SDS	sodium dodecyl sulfate
TAF	TBP-associated factor
TBP	TATA-binding protein
TBS	Tris-buffered saline
TLC	thin layer chromatography
TRNA	transfer ribonucleic acid
UDP	uridine diphosphate

CHAPTER 1

Introduction

1.1 Glycosphingolipids and Gangliosides

Glycosphingolipids (GSLs) are a typical component of eukaryotic cell membranes, anchored in the outer leaflet of the bilayer by a hydrophobic ceramide moiety. Attached to the ceramide and extending into the extracellular space is a hydrophilic oligosaccharide chain which can vary in length and composition. Such variation in the type, number and linkage of sugar residues in the oligosaccharide chain gives rise to the wide range of naturally occurring GSLs (Kolter & Sandhoff, 1998). GSLs form cell- and species-specific patterns on the surface of cells that change with cell growth, differentiation, viral transformation and oncogenesis (Hakomori, 1981). In addition, cell surface GSLs interact with toxins, viruses and bacteria (Karlsson, 1989), as well as with membrane-bound receptors and enzymes (Schnaar, 1991). Gangliosides are a group of complex GSLs which contain one or more sialic acid residues on their oligosaccharide chain (Fig. 1.1). Gangliosides are present in all tissues, but are highly enriched in the central nervous system (Gravel et al., 1995). The terminal location of and negative charge associated with sialic acid has implicated gangliosides in numerous cellular functions (Varki, 1997). Indeed, many of the cellular functions of complex GSLs can be attributed to sialic acid-containing gangliosides. Some of these functions include embyrogenesis, neuronal and leukocyte differentiation, cell adhesion and signal transduction (Zeller & Marchase, 1992). Gangliosides have also been shown to act as inhibitors to numerous intermolecular and intercellular interactions, while also acting as ligands for a wide variety of lectins of animal, plant, and microbial origin (Varki, 1997).

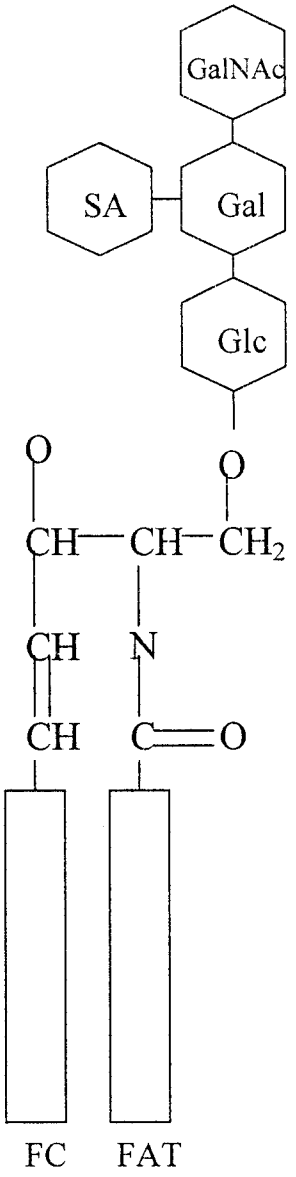


Figure 1.1 Structure of ganglioside GM2. GalNAc = N-acetylgalactosamine; Gal = Galactose; SA = Sialic Acid; Glc = Glucose; FC = Fatty Chain; FAT = Fatty Acid Tail.

Ganglioside biosynthesis (reviewed in Sandhoff & Van Echten, 1993) begins on the cytosolic face of the endoplasmic reticulum (ER) with the condensation of L-serine and palmitoyl-CoA, which is catalyzed by the pyridoxal phosphate-dependent serine palmitoyltransferase (SPT), yielding 3-dehydrosphinganine. This ketone is rapidly reduced to sphinganine by a NADPH-dependent reductase, followed by the addition of an amide-linked fatty acid and the introduction of a 4-*trans* double bond, ultimately yielding ceramide. Ceramide is the common precursor of GSLs and sphingomyelin and is transported to the Golgi apparatus by unknown mechanisms. Once in the Golgi, ceramide glucosyltransferase transfers a glucose residue from UDP-glucose to ceramide, yielding glucosylceramide (GlcCer). Ceramide glucosyltransferase has been shown to be accessible from the cytoplasmic face of Golgi vesicles, whereas the following step in ganglioside biosynthesis, the addition of galactose to the oligosaccharide chain by galactosyltransferase I, occurs on the luminal side of the Golgi. This implicates a membrane translocation of glucosylceramide, possibly by an as yet uncharacterized "flippase". The action of galactosyltransferase I results in the formation of lactosylceramide (LacCer), the common precursor of all GSL families. The production of complex gangliosides results from the sequential addition of monosaccharide and sialic acid residues to the growing oligosaccharide chain. The addition of these residues is catalyzed by membrane-bound glycosyltransferases, which have been shown to be restricted to the luminal face of the Golgi apparatus. As a result the growing glycan chain is oriented extracytosolically, an orientation topologically equivalent to the situation in the plasma membrane, where the sugar residues of complex gangliosides project into the extracellular space. The addition of the first carbohydrate residues to LacCer is

performed by highly specific sialic acid transferases, and results in the formation of the four series of complex gangliosides. These different series are characterized by the presence of no (0-series), one (a-series), two (b-series), or three (c-series) sialic acid residues linked to the inner galactose moiety. In adult human tissues, gangliosides from the 0- and c-series are found only in trace amounts. This report shall focus mainly on gangliosides from the a-series, in particular ganglioside GM2 (Fig. 1.1), which is most abundant (although at relatively low levels compared to GM1) in neuronal cells (Mahuran, 1991). Once ganglioside biosynthesis is completed in the Golgi, the complete gangliosides are transported to the plasma membrane by vesicle flow.

As part of the endocytotic turnover of plasma membrane components, gangliosides are routinely routed to the lysosome for degradation via the endosomal compartments (reviewed in Kolter and Sandhoff, 1998). Conflicting views exist, however, regarding the precise mechanism of this delivery. In the first of these theories, plasma membrane components are thought to reach the lysosomal compartment via the early and late endocytic reticulum. A series of budding and fusion events are believed to occur through the endosomal compartment, and the membrane components ultimately reach the lysosomal compartment as part of the lysosomal membrane. Because the inner leaflet of the lysosomal membrane is protected by a thick glycocalix composed of glycoproteins, this model fails to explain how the former plasma membrane components can be selectively degraded without the destruction of the lysosomal membrane. The alternative model for the topology of endocytosis has plasma membrane components passing through the endosomal compartment as intraendosomal vesicles that later become intralysosomal vesicles

upon reaching the lysosome (Sandhoff and Van Echten, 1993). The vesicles are formed initially in the early endosome by the selective invagination of endosomal membrane regions enriched with plasma membrane components. The surrounding endosome passes through the typical endocytic pathway by successive membrane fusion and fission events, while the intraendosomal vesicles are carried as passengers. When the vesicle reaches the lysosome, glycoconjugates originating from the outer leaflet of the plasma membrane face the lysosol on the outer leaflet of the intralysosomal vesicle membrane, and are thus unobstructed by a glycocalix and readily accessible by degrading enzymes (Sandhoff and Van Echten, 1993).

Within the lysosome, degradation of gangliosides occurs by the stepwise action of specific acid exohydrolases. Degradation of a-series gangliosides begins with the removal of the terminal galactose residue from ganglioside GM1 by the action of β-galactosidase, yielding ganglioside GM2. The following step involves the removal of N-acetylgalactosamine (GalNAc) from ganglioside GM2 by the action of β-hexosaminidase A (Hex A) and a lysosomal ganglioside binding protein, the GM2 activator. In contrast to the membrane-bound glycosyltransferases involved in ganglioside biosynthesis, Hex A is a water-soluble enzyme that acts on substrates on the membrane surface only if they extend far enough into the aqueous space. Because the oligosaccharide group on ganglioside GM2 is too short to be reached by Hex A, it requires the GM2 activator protein to bind and extract ganglioside GM2 from its membrane and present it to Hex A for degradation. The removal of GalNAc from GM2 yields ganglioside GM3, which in turn has its sialic acid residue removed by lysosomal sialidase, yielding LacCer. LacCer is ultimately degraded into its

component molecules, which can re-enter the biosynthetic pathway to form new GSLs.

A deficiency in any of the enzymes or activator proteins involved in the sequential degradation of gangliosides results in accumulation of its corresponding substrate within the lysosome. The massive accumulation of ganglioside in the lysosomes caused by such metabolic deficiencies can lead to a wide range of clinical symptoms, and thus make up a family of inherited metabolic disorders known as gangliosidoses. A deficiency in any of the proteins responsible for the degradation of ganglioside GM2 results in its accumulation within the lysosome. This disease is known as GM2 gangliosidosis, and it will be the focus of the following section.

1.2 GM2 Gangliosidoses

In 1881 a British ophthalmologist named Warren Tay first observed a cherry-red spot on the retina of a one-year-old boy with physical and mental retardation. A few years later the American neurologist Bernard Sachs noted the swollen neurons that were characteristic of the disorder that would come to be known as Tay-Sachs disease. Sachs also recognized the prevalence of the disease in Jewish babies of Eastern European origin. In the 1930s, the German biochemist Ernst Klenk identified the cause of the swollen neurons as the abnormal storage of a new group of acidic glycolipids he named gangliosides. It was not until 1962 that Svennerholm identified ganglioside GM2 as the specific neuronal storage component in Tay-Sachs disease (Gravel et al., 1995).

In 1969, Okada, O'Brien, and Sandhoff discovered β-hexosaminidase A activity was absent in Jewish patients with Tay-Sachs disease. This discovery resulted

in biochemical diagnosis of the disease and widespread carrier screening in Jewish populations. In addition, Sandhoff also recognized patients of non-Jewish heritage with identical symptoms but instead showing deficiency in both Hex A and a related enzyme, Hex B; he also noted patients missing neither isoenzyme. In the years that followed, Hex A was revealed to be a heterodimer composed of non-identical α- and β-subunits, while Hex B (responsible for the lysosomal degradation of ganglioside GA2) was a homodimer made up of familial β-subunits; a defect in the α-subunit would therefore cause Hex A deficiency, while a defect in the β-subunit would result in deficiency of both Hex A and Hex B. The disease associated with deficiency of both hexosaminidase enzymes came to be known as Sandhoff disease. It was also found that the protein GM2 activator played a vital role in ganglioside GM2 degradation, and that a defect in this protein caused similar storage of GM2 despite the presence of functional Hex A. This rare form of GM2 gangliosidosis came to be known as the AB variant, so named for the presence of normal levels of functional Hex A and Hex B. In order to describe all three forms of ganglioside GM2 storage disorders, the comprehensive term GM2 gangliosidosis was introduced by Suzuki and Chen (Gravel et al., 1995).

The GM2 gangliosidoses show great variability in clinical phenotype (reviewed in Mahuran, 1991, and Gravel et al., 1995). Patients are normally characterized on the basis of their age at onset of clinical symptoms. Generally, the earlier the onset of symptoms the more severe the resulting disease. As discussed above, the three forms of GM2 gangliosidoses result from mutations associated with any of the three gene products necessary for GM2 hydrolysis. Different mutations in

these genes lead to various levels of residual function, however, and the varying clinical phenotypes result from these different mutations. All three disorders follow a similar course and have similar symptoms, particularly from a neuropathological perspective, thus the three clinical phenotypes described below are applicable to all three forms of GM2 gangliosidoses.

Infantile Acute/Infantile onset phenotype

The classical forms of Tay-Sachs disease and Sandhoff disease refer to the infantile acute phenotypes, and are caused by mutations leading to the profound deficiency or complete absence of enzyme activity. Appearing normal at birth, affected infants begin to show symptoms of mild motor weakness at 3 to 5 months of age, accompanied by an exaggerated startle reaction. Progressive weakness and the inability of the infant to crawl or sit unsupported by 6 to 10 months of age often provoke parents to seek medical attention. Opthalmoscopic examination at this time would likely reveal the marked prominence of the fovea centralis, the so-called "cherry red spot" found in virtually all cases of infantile Tay-Sachs and Sandhoff disease.

The disease progresses rapidly after 8 to 10 months of age, with the child becoming increasingly unresponsive to surroundings. There is an accelerated loss of vision, enlargement of the head, and seizures, which become more frequent and severe with time. The child deteriorates towards a completely vegetative state by 2 years of age with death following before the age of four, typically from bronchopneumonia. Neuropathological features include widespread marked neuronal storage throughout the neuroaxis, with swollen storage neurons containing characteristic neuronal

inclusions, membranous cytoplasmic bodies (MCBs). In the severe infantile cases, the accumulation of ganglioside GM2 alone may amount to 12 percent of dry brain weight, and the resulting storage granules may fill the entire cytoplasm of a neuronal cell body (Gravel et al, 1995).

It should be noted that while Tay-Sachs disease and Sandhoff follow this course, patients with Sandhoff disease also show symptoms due to Hex B deficiency and the subsequent accumulation of ganglioside GA2. These symptoms involve peripheral tissues, and include hepatosplenomegaly and dystosis multiplex. The rare AB variant (GM2 activator deficient) has only ever been observed to follow an acute infantile phenotype.

Juvenile/Late infantile phenotype

The juvenile forms of the GM2 gangliosidoses are the manifestations of severely reduced, but not entirely absent, enzyme activity. Because of this residual enzyme activity, the juvenile form does not present until 2-6 years of age. The juvenile phenotype is characterized by incoordination, developmental regression, progressive dementia, ataxia, and seizures that increase in frequency towards the end of the first decade of life. Loss of vision occurs much later than in the infantile form, and the cherry-red spot is not often observed. The child progresses towards a vegetative state by 10-15 years of age with death following soon after.

Adult Onset/Chronic phenotype

The adult onset form is the most clinically heterogeneous type with extreme variability of symptoms even between members of the same family (Argov and

Navon, 1984). Severity of symptoms depends on the specific level of residual enzyme activity, though all variants remain related by the widespread involvement of the central nervous system. While the clinical differentiation between Hex A deficiency and combined Hex A/Hex B deficiency in the infantile and juvenile forms is generally not possible, the adult onset form is typically due to deficiency of Hex A. The symptoms include progressive dystonia, spinocerebellar degeneration, motor neuron disease, and psychosis. Psychiatric problems are the most common manifestation of the adult onset form, affecting as many as 40% of patients. These problems include schizophrenia, hallucinations, paranoia, and recurrent psychotic depression. Patients with chronic GM2 gangliosidosis are capable of living a normal lifespan. Interestingly, individuals who are completely asymptomatic, but have very low Hex A levels by standard assay procedures, have also been described (Conzelmann et al., 1983).

The pathogenic mechanisms that lead to the malfunctioning of neuronal circuitry and neurodegeneration in ganglioside storage diseases are not yet understood. Although the storage compounds themselves are normal, non-toxic membrane components, the excessive accumulation is likely to interfere with intracellular transport and other activities, ultimately leading to unscheduled apoptosis (Huang et al., 1997). It has also been suggested that the model of neurodegeneration in these disorders includes inflammation as a major factor leading to the loss of neurons. Indeed, recent work involving gene expression profiling in Tay-Sachs cerebral cortex cells has provided evidence consistent with this view (Myerowitz et al., 2002)

The entire primary structure of the α- and β-subunits have been determined from their respective cDNA clones (Myerowitz and Proia, 1984; O'Dowd et al., 1985) and found to be 60% homologous (Korneluk et al., 1986). The genes encoding the α- and β-subunits, *HEXA* (chromosome 15q23-24) and *HEXB* (chromosome 5q15), respectively, are each made up of 14 exons and 13 introns. The homology that exists among the intron/exon junctions of the two genes demonstrates that the genes likely arose from a common ancestor. The demonstration of this common evolutionary origin was not surprising given the biochemical properties shared by the α- and β-subunits; dimer formation is required for either subunit to become active, and both α- and β-subunits contain active sites which are able to hydrolyse many of the same natural and synthetic substrates. However, only the active site in the α-subunit is able to hydrolyse ganglioside GM2 (Mahuran, 1991). Furthermore, elements of both α- and β-subunits in Hex A are necessary for GM2 activator/GM2 binding, with the α-subunit supplying the catalytic site for GM2 hydrolysis and the β-subunit conferring stability to the dimer (Mahuran, 1991).

Characterization of *HEXA* and *HEXB* has made it possible to identify the specific nucleotide changes, i.e., the genotypes, that result in Tay-Sachs and Sandhoff disease. Several types of mutations have been identified as being causative of GM2 gangliosidosis, including partial gene deletions, small insertions or deletions, and base substitutions (reviewed in Mahuran, 1991, and Gravel et al., 1995). Many of the mutations are highly population specific, displaying much higher prevalence in certain ethnic groups. In general, those mutations that result in absent or unstable mRNA lead to the classic, infantile phenotypes. Less severe point mutations that result in the

production of stable mRNA, and subsequent residual enzyme activity, are typically associated with the later-onset forms. Relating these genotypes to previously defined clinical and biochemical phenotypes has enhanced our understanding of the disease mechanism and localized potentially important functional areas within the α- and β-subunits (Mahuran, 1991).

Over 70 mutations of *HEXA* have been identified, with the vast majority being associated with the infantile acute phenotype. The first mutation identified was a 7.6 kb deletion at the 5' end of the *HEXA* gene of a French Canadian patient, resulting in an mRNA-negative phenotype (Myerowitz and Hogikyan, 1987). This discovery was followed by the identification of three mutations that account for 98% of cases of Tay-Sachs disease in Ashkenazi Jews, a group that displays particularly high incidence of the disease (carrier rate of 1 in 30, compared to 1 in 300 in non-Jewish populations). The first of these mutations is a 4 bp insertion in exon 11 resulting in a premature stop codon; this mutation accounts for 81% of the mutations in the Ashkenazi Jewish population (Myerowitz and Costigan, 1988). The other two mutations, one a splice junction point mutation that results in abnormally spliced mRNAs, the other a point mutation in exon 7 that results in inhibited subunit association, account for the remaining 16% and 3%, respectively (Myerowitz, 1988; Navon and Proia, 1989). Also present in Jewish populations are "pseudodeficiency alleles", mutations that lead to an apparent deficiency in Hex A but whose carriers are nonetheless healthy. The α-subunit that is produced from such mutations is inactive towards the synthetic substrates used for diagnosis, but performs ganglioside GM2 hydrolysis in the low-normal range (Gravel et al., 1995).

Mutations of the *HEXB* gene have been studied far less extensively than those of *HEXA*, though at least 12 different alleles resulting in Sandhoff disease and its variants have been identified. The most common *HEXB* mutation is a 16 kb deletion spanning the promoter, exons 1 through 5, and part of intron 5. Homozygosity for this allele is associated with infantile Sandhoff disease (Neote et al., 1990). Sandhoff disease alleles display no apparent predilection for any specific ethnic group. Point mutations within the structural gene of the GM2 activator, *GM2A*, have been identified in four patients with the AB variant of GM2 gangliosidosis, leading to premature degradation of the gene product (Schepers et al., 1997). The mutations were discovered in patients whom displayed the infantile acute phenotype, yet were found to have normal levels of Hex A and Hex B activity when measured with synthetic substrates. All GM2 gangliosidoses exhibit an autosomal recessive pattern of inheritance, with heterozygote carriers of diseased alleles being completely asymptomatic (Gravel et al., 1995).

There is no known cure for any of the GM2 gangliosidoses, owing to their inherent genetic basis. However, the observation of reduced Hex A activity in serum, leukocytes, fibroblasts, and tears of heterozygotes for Tay-Sachs mutations has led to the development of simple, inexpensive, and highly accurate methods for carrier identification. These simple tests have been in widespread use since the early 1970s, when large-scale screening of high-risk Jewish communities began throughout North America. In fact, Tay-Sachs disease was the first genetic condition for which community-based screening methods were implemented, and has since served as a model for screening programs for other disorders (Kaplan, 1998). These screening efforts, along with genetic counselling and prenatal diagnosis, have led to a near 90%

decrease in Tay-Sachs births in the Ashkenazi Jewish population. Non-Jewish populations, however, show a relatively consistent occurrence of Tay-Sachs, due to the lack of screening of low-risk groups (Gravel et al., 1995).

In an effort to keep patients as comfortable as possible, current treatment of the GM2 gangliosidoses focuses mainly on management of symptoms. In patients with the infantile acute phenotype, treatment involves providing adequate nutrition and hydration, management of infectious disease, and seizure control. In treating older patients with chronic GM2 gangliosidosis, conventional antipsychotic or antidepressant therapy may be employed, though results are inconsistent.

Attempts to reduce the amount of stored ganglioside in the GM2 gangliosidoses have been met with limited success. One of the treatments most often attempted has been enzyme replacement therapy (ERT), but the blood-brain barrier has remained a major obstacle – intravenous injection of purified human Hex A results in rapid clearance from the circulation with no transfer of infused enzyme into the central nervous system. Attempts at circumventing the blood-brain barrier have also led to discouraging results – brain biopsies of Tay-Sachs patients injected intrathecally with purified Hex A have shown no significant changes in brain ganglioside content. It has been suggested that bone marrow transplantation might offer a potentially successful therapeutic approach to the GM2 gangliosidoses, but it remains unclear whether storage disorders involving the central nervous system would benefit from this therapy, which, moreover, has a high mortality and morbidity (Gravel et al., 1995). Potential treatments may also exist with the use of gene therapy, though this technology remains very much in the experimental stage.

An encouraging potential therapeutic approach exists, however, in the continuing development of substrate deprivation therapy. This strategy is based on the partial inhibition of ceramide glucosyltransferase, which catalyzes the first step in GSL biosynthesis. This would reduce the levels of GSLs synthesized to such a degree as to allow any residual enzyme activity to prevent GSL accumulation. If the rate of accumulation can be slowed sufficiently to prevent the cytotoxic threshold of ganglioside storage from being reached, the patient may exhibit a greatly slowed rate of disease progression, or may avoid symptomatic disease altogether. Much of the research in this field has been done using a specific inhibitor of ceramide glucosyltransferase, the *N*-alkylated imino sugar *N*-butyldeoxynojirimycin (NB-DNJ). This compound is water soluble and noncytotoxic over a broad range of concentrations *in vitro* and *in vivo*. Its oral administration to healthy mice results in GSL depletion in multiple organs, without causing any overt pathology in the treated animals (Platt et al., 1997). Furthermore, NB-DNJ has been shown to delay the onset of symptoms and increase life expectancy in a mouse model of Sandhoff disease (Jeyakumar et al., 1999), as well as deplete lysosomal storage of ganglioside GM2 in a mouse model of Tay-Sachs disease (Platt et al., 1997). Because of its reliance on residual enzyme activity to prevent ganglioside accumulation, substrate deprivation therapy alone may only be clinically applicable to the juvenile and chronic forms of GM2 gangliosidosis. If sufficient advances in enzyme augmentation therapy can be made, however, substrate deprivation may be useful in combination with other therapies in the treatment of infantile forms of the disease.

As mentioned above, mouse models have been important tools in the research of and the development of potential therapies for the GM2 gangliosidoses. The

development of *HEXA* and *HEXB* knockout mice as *in vivo* models of human Tay-Sachs disease and Sandhoff disease, respectively, by targeted disruption of the genes (Phaneuf et al., 1996) was an invaluable step in the field of GM2 gangliosidosis research, with the use of the models leading to numerous advances. The first such advance came immediately following the development of the two mouse models with the observation that the mice, which were engineered to mimic phenotypically similar human disorders, displayed drastically different phenotypes (Phaneuf et al., 1996). While the Sandhoff model mice showed physical and neurodegenerative symptoms consistent with the human disorder, followed by death within six weeks of onset of symptoms, the Tay-Sachs model mice showed no apparent difference in size, behaviour, or reproductive ability compared to wild-type litter mates up to 1 year. Immunohistochemistry revealed that both models did indeed accumulate ganglioside GM2 and that both displayed the characteristic lysosomal inclusions in their neuronal cells. The Sandhoff mice, however, showed far more extensive and widespread storage of ganglioside, with the greatest differences in distribution appearing in the cerebellum, brainstem, and spinal cord. These surprising results prompted the researchers to propose a biochemical model by which *Hexa -/-* mice were able to escape disease by keeping ganglioside GM2 stores below toxic levels. The model hypothesizes that in mice, another lysosomal enzyme, sialidase, is able to cleave the sialic acid residue from GM2, yielding ganglioside GA2, which is then further degraded by Hex B to lactosylceramide (Fig. 1.2). This model was validated by a later study of ^{3}H-GM1 catabolism in embryonic fibroblasts cultured from *Hexa -/-* and *Hexb -/-* mice which further demonstrated the dominance of the GA2 pathway (Sango et al., 1995). It remained unknown at this point, however, whether the sialidase-

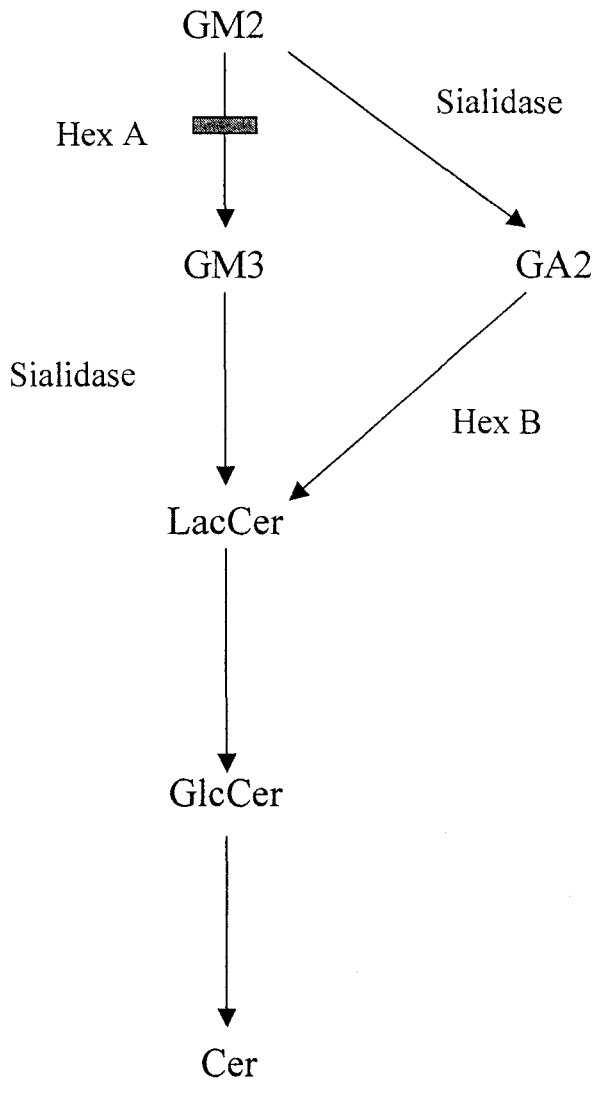


Figure 1.2 Pathways of GM2 ganglioside catabolism to ceramide. The vertical scheme is the standard pathway known in human tissues where GM2 is acted upon by Hex A followed by sialidase. The sialidase "bypass", predominant in mouse tissues, shows GM2 acted upon by sialidase followed by Hex B. The shaded box illustrates the metabolic block in Tay-Sachs disease (Hex A deficiency). LacCer = lactosylceramide; GlcCer = glucosylceramide; Cer = ceramide.

mediated bypass in mice was due to a greater affinity of mouse sialidase for ganglioside GM2, or rather to an increased level of sialidase expression or activity. This question was soon answered by a study in which sialidase levels were elevated in human Tay-Sachs neuroglia cells by transfection, resulting in the depletion of stored ganglioside GM2 (Igdoura et al., 1999). Since increasing the level of sialidase in human Tay-Sachs cells appeared to result in a bypass pathway of GM2 degradation similar to that observed in the Hex A knockout mouse, it became apparent that increasing sialidase levels in human Tay-Sachs patients might serve as an effective therapy for the disease. Before such a therapy can be evaluated, however, a better understanding of human lysosomal sialidase, a poorly characterized protein, and its regulation in human cells must be sought out.

1.3 Sialidases

The sialidases are a family of hydrolytic enzymes that cleave terminal sialic acid moieties from glycoproteins, oligosaccharides, and glycolipids. The action of sialidases on their target molecules is associated with numerous important biological reactions such as antigenic expression and recognition of cell surface receptors (Saito and Yu, 1995; Reuter and Gabius, 1996). While the biochemistry and *in vivo* function of viral and bacterial sialidases are well characterized, the biological role of mammalian sialidases remains unclear. Multiple mammalian sialidase genes have been discovered and cloned to date, including those encoding lysosomal (Bonten et al., 1996; Carrillo et al., 1997; Igdoura et al., 1998; Milner et al., 1997; Pshezhetsky et al., 1996, 1997), cytosolic (Ferrari et al., 1994; Miyagi et al., 1993; Monti et al., 1999), and plasma membrane-bound forms (Miyagi et al., 1999; Wada et al., 1999).

These different forms of mammalian sialidase differ in both subcellular location and substrate specificity.

Human lysosomal sialidase is a glycoprotein that exists in two isoforms of 44kD and 48kD. It is only active when part of a lysosomal multienzyme complex with β-galactosidase and the protective protein/cathepsin A (PPCA), itself a serine carboxypeptidase (d'Azzo et al., 1982; Verheijen et al., 1982). It is believed that PPCA interacts with sialidase shortly after sialidase synthesis in the ER and that it is required for targeting of the complex to the lysosome (van der Spoel et al., 1998), though the precise mechanism by which PPCA regulates sialidase activity is unknown. Because sialidase is poorly phosphorylated and its mannose 6-phosphate marker is not functional in receptor-mediated endocytosis of the enzyme, PPCA is believed by some to act as an auxiliary transport protein for sialidase, with sialidase acquiring full enzymatic activity in mature lysosomes only when bound to PPCA (Bonten and d'Azzo, 2000). In contrast, others have reported sialidase reaching the lysosome in the absence of functional PPCA, though it was rapidly degraded (Vinogradova et al., 1998). Thus, the interaction of sialidase and PPCA within the multienzyme complex has yet to be properly characterized. In humans, deficiency of sialidase is associated with two distinct disorders of metabolism: sialidosis, caused by a defect in the sialidase gene, and galactosialidosis, in which sialidase deficiency exists due to a defect in the gene encoding PPCA.

The human lysosomal sialidase gene has been mapped to chromosome 6p21 (Pshezhetsky et al., 1997), while the mouse version has been mapped to chromosome 17 (Womack et al., 1981). Both the human and mouse sialidase genes are approximately 4 kilobases long, structurally similar, and encode proteins which are

highly homologous (80%) in terms of amino acid structure (Igdoura et al., 1998). As mentioned above, mouse sialidase appears to be expressed at levels adequate to facilitate the sialidase-mediated bypass of Hex A deficiency, as Hex A knockout mice are asymptomatic for the Tay-Sachs phenotype. Human Tay-Sachs patients, on the other hand, appear unable to bypass their Hex A deficiency with sialidase, and suffer from the neurogenerative disease associated with excessive storage of ganglioside GM2 in the CNS. It is therefore believed that the difference between human and mouse Hex A deficient systems lies in the regulation of sialidase expression, which is poorly understood in both species.

1.4 Regulation of eukaryotic gene expression and preliminary comparison of human and mouse lysosomal sialidase promoters

There are several key points at which gene expression is controlled in eukaryotic cells (reviewed in Ogbourne and Antalis, 1998; Struhl, 1999) including activation of gene structure, transcription initiation, termination of elongation, nuclear RNA processing, mRNA transport, mRNA translation, and mRNA stability. Despite the importance of factors controlling RNA synthesis, structure and stability in the regulation of gene expression, it is generally accepted that the most crucial regulatory events in gene expression occur at initiation. Before initiation of gene transcription can occur, however, the highly organized and densely packed structure known as chromatin, which physically inhibits the binding of initiation factors to DNA, must be activated. The precise mechanism of the unravelling and priming of chromatin is unknown, but it is believed to be regulated by histone proteins and acetylation or methylation events.

As mentioned, initiation of transcription is the most crucial event in regulation of gene expression. The key enzymes in the initiation of transcription are RNA polymerases, a family of large proteins composed of 8-14 subunits and possessing a molecular mass of 500 kDa or more. There are three different RNA polymerases present in eukaryotic cells, each with a specific function: RNA polymerase I, which is localized to the nucleolus, is responsible for the transcription of rRNA; RNA polymerase II is responsible for the transcription of genes and the synthesis of mRNA; and RNA polymerase III synthesizes tRNA, 5 S RNA and small nuclear RNA. As the focus of this paper is on the transcription of genes, the remainder of this section will focus on the mechanisms of RNA polymerase II function and regulation.

RNA polymerase II is completely dependent of auxiliary transcription factors to allow it to initiate transcription. These auxiliary transcription factors are referred to as the general factors, and they complex with RNA polymerase II at the initiation site to form what is known as the basal transcription apparatus. The basal transcription apparatus forms in a highly regulated and defined order, resulting in a complex with a molecular mass greater than 2500 kDa. The first general factor to bind to the transcription initiation site is the TATA-binding protein (TBP), and it does so at an 8 bp consensus sequence (TATAAAA) known as the TATA box. The TATA box is present in most eukaryotic promoters as a component of the core promoter. Located approximately 20 to 45 bp upstream of transcriptional start, the core promoter is sufficient to signal transcription initiation. Once TBP binds to the TATA box, a group of proteins known collectively as TBP-associated factors (TAFs) bind to it, resulting in a complex known as transcription factor IID (TFIID). The basal transcription apparatus formation continues with the recruitment of transcription factor IIA

(TFIIA), resulting in the TFIIA-TFIID complex. This intermediate complex then undergoes a conformational change that facilitates the binding of TFIIB, followed by the binding of the pre-assembled TFIIF-RNA polymerase IIa (non-phosphorylated form). TFIIE then interacts directly with RNA polymerase IIa and subsequently recruits TFIIF to the C-terminal domain of RNA polymerase IIa. A large aggregate of 20 or more proteins, known as mediator, then assembles at the C-terminal domain. The C-terminal domain of RNA polymerase IIa is finally phosphorylated, inducing the removal of TFIIB, TFIIE, and TFIIH from the complex, which in turn permits RNA polymerase IIo (the phosphorylated form of RNA polymerase II) to commence transcription. Another model of basal transcription apparatus assembly has a large multisubunit complex known as the RNA polymerase II holoenzyme, consisting of RNA polymerase II, TFIIB, TFIIH and mediator, associating independently of a promoter. It is believed that the response of this pre-assembled complex to core promoter elements may result in faster transcription initiation.

The presence of a core promoter sequence and assembly of the basal transcription apparatus is all that is required to initiate transcription of any given gene. However, the levels of transcription that are achieved at such a promoter are minimal, and require the presence of upstream factors to increase them. These upstream factors fall into two categories based on the effect they have on the rate of transcription. As their name suggests, enhancers are responsible for increasing rates of transcription. Many enhancers and their corresponding proteins have been identified, and it has been found that many enhancer elements are common to the majority of promoters. For example, the CCAAT box, so named for its consensus sequence of 5' GGCCAATCT 3', is an enhancer element generally found around 75 bp upstream of the transcription

initiation site. There are also a large number of enhancer-like elements known as response elements that increase rates of transcription in response to external stimuli. These motifs are usually found further upstream than elements such as the CCAAT boxes, but generally exert their effects on gene transcription in the same manner. Enhancers and the proteins that bind to them act to increase gene expression by recruiting members of the basal transcription apparatus, such as mediator, to the initiation site.

A more complex and mechanistically diverse group of elements, known as silencers, are responsible for decreasing rates of transcription. Silencers were initially defined as upstream sequence elements capable of repressing promoter activity by recruiting transcription factors which would in turn carry out a specific function. Only recently has become known that numerous types of silencers exist that are capable of affecting many aspects of gene regulation, such as cytoplasmic retention of transcription factors, activity of positive-acting transcription factors, chromatin structure, intron splicing, and general factor assembly to ultimately down-regulate transcription. Among the various possible mechanisms of transcription repression, two distinct functional types of silencers have emerged: classical silencers, position-independent elements which direct an active repression mechanism, usually by interfering with general factor assembly; and NREs, non-classical, position-dependent elements that direct a passive repression mechanism by interfering with upstream enhancer elements.

The proteins that interact with silencers are known as repressors. Though many silencers and their corresponding repressors have been identified, the mechanisms of silencer and repressor interaction, and subsequent transcriptional

downregulation, remain poorly understood. It is known, however, that the mechanisms are varied and complex – some repressors have been found to interact with multiple binding sites, others are known to act as both repressors and activators, possibly in a cell type- or promoter-specific manner.

Analysis of ~ 2.9 kb of sequence upstream of the gene for human lysosomal sialidase with MatInspector, a common transcription element identification software, revealed the presence of numerous common enhancers and silencers. In addition, a BLAST alignment of the human and mouse lysosomal sialidase promoters revealed numerous regions of high homology (Fig. 1.3), indicating that both human and mouse lysosomal sialidase genes may share similar transcriptional regulation systems. One of the elements shared by both the human and mouse lysosomal sialidase promoters is the putative binding site for the CCAAT-displacement protein (CDP), a transcription factor believed to act mainly as a repressor. Transient transfection assays with promoter deletion-reporter constructs of the mouse lysosomal sialidase promoter have shown the importance of the CDP-binding site in promoter activity; a construct lacking the site showed a many-fold increase in reporter gene activity over a construct of similar length with the site intact (Champigny, unpublished data). Due to its apparent significance in the regulation of both human and mouse lysosomal sialidase expression, a more thorough discussion of CDP is warranted.

1.5 The CCAAT-Displacement Protein (CDP)

The CCAAT-displacement protein (CDP) is the human homologue of the Cut homeodomain protein, a transcription factor known primarily for its importance in the

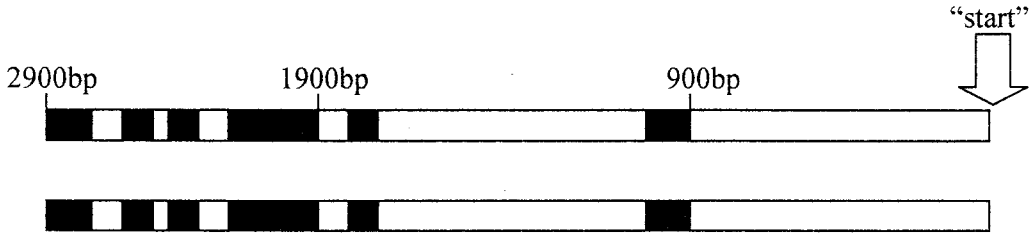


Figure 1.3 Schematic of the alignment of human and mouse lysosomal sialidase promoters. Black boxes indicate regions of high homology.

determination and maintenance of cell-type specificity in *Drosophila melanogaster* (reviewed in Nepveu, 2001). The CDP/Cut family constitutes a unique group of homeoproteins that are highly conserved among higher eukaryotes. Indeed, several CDP homologues have been isolated from several different species, including human (Neufeld et al., 1992), dog (Andres et al., 1992), mouse (Valarche et al., 1993), and rat (Yoon and Chikaraishi, 1994). The gene for human CDP, *CUTL1*, encodes a 200 kDa protein and has been mapped to chromosome 7q22 (Scherer et al., 1993; Lemieux et al., 1994). Interestingly, rearrangements or deletions of 7q22 occur frequently in various cancers. While studies in *Drosophila* have shown the importance of Cut in developmental processes, mammalian CDP is known primarily as a transcriptional repressor which, as its name suggests, operates by competing with activators for the occupancy of the CCAAT enhancer element.

The bulk of the results in mammals have suggested that CDP/Cut expression or activity might be restricted to proliferating cells. For example, CDP/Cut binding activity has been found to be downregulated during development of the fetal liver (van Wijnen et al., 1991) as well as during development of cells in the myeloid lineage (van Wijnen et al., 1989) and osteoblast differentiation (Holthuis et al., 1990). Consistent with the ubiquitous downregulation of CDP/Cut binding activity in many mammalian cell types, many of the identified targets of CDP/Cut are genes that are repressed in proliferating precursor cells and are turned on as cells become terminally differentiated and CDP/Cut binding activity ceases (Nepveu, 2001). There are also a number of observations that suggest a role for CDP/Cut in cell cycle progression, as CDP/Cut DNA binding activity has been found to oscillate during the cell cycle, reaching its maximum during S phase (Coqueret et al., 1998). It is not known,

however, whether the decrease in CDP/Cut DNA binding activity observed in mammals as cells differentiate is due to a drop in expression or to some post-translational modifications. Recent evidence suggests that it is the latter situation, for CDP/Cut has been observed to undergo a number of such modifications (Coqueret et al., 1996; Moon et al., 2001; Moon et al., 2002), and the isolation of a neuronal specific CDP, Cux-2, from the brain of adult mice indicates the persistent expression of CDP in terminally differentiated mammalian cells (Quaggin et al., 1996).

In addition to its role as a transcriptional repressor, there is evidence that members of the CDP/Cut family may also function as transcriptional activators. A number of groups have identified binding sites for CDP/Cut in the regulatory sequences of genes encoding for proteins that have their peak expression either during or closely preceding DNA replication, the time at which CDP/Cut has its highest DNA binding activity (Barberis et al., 1987; el-Hodiri and Perry, 1995; van Wijnen et al., 1996; Kim et al., 1997). Moreover, CDP/Cut was found to be the DNA binding component of the promoter complex HiNF-D, which is believed to contribute to the transcriptional induction of several histone genes at the G1/S phase transition of the cell cycle (van Wijnin et al., 1996). The molecular basis for the alternate action of CDP/Cut on different promoters is unknown at this time, though it has been suggested that it may be regulated through interaction with other proteins. For example, CDP/Cut alone could not activate a reporter plasmid corresponding with the gene for tyrosine hydroxylase, but it could when co-transfected with the rat ITF2 transcription factor (Yoon and Chikaraishi, 1994). It is therefore possible that CDP/Cut can function either as a transcriptional repressor or activator in either a cell type- or promoter-specific manner, based on the given regulatory environment.

Members of the CDP/Cut family are unique in that they contain multiple DNA-binding domains: the Cut homeodomain (CHD); and three 'Cut repeats' – CR1, CR2, and CR3 (Fig. 1.3). The presence of these four domains is characteristic of the CDP/Cut family, and they confer a distinctive ability to bind to DNA and regulate gene expression. A recent study found that these domains bind DNA most effectively when in pairs, and, as such, they concluded that the structurally adjacent CHD and CR3 are responsible for the stable binding of CDP to its putative binding sequence, ATCGAT, while CR1 and CR2 are involved in transient binding to nearby CCAAT sites and subsequent displacement of CCAAT box-binding activators (Moon et al., 2000). However, the ability of various combinations of the Cut repeats and the CHD to effectively bind DNA suggests a more complex pattern of DNA binding – the combinations CR1-CHD and CR2-CHD were both able to bind DNA, despite the apparent unlikelihood that such combinations would arise in the context of the full-length protein. Moreover, while numerous studies have implicated ATCGAT as the binding site for CDP, others (Zhu and Dudley, 2001) have observed the ability of CDP to bind to various short DNA sequences; these observations also indicate a multifaceted pattern of DNA binding. It is possible that variations in the DNA binding properties of CDP/Cut are due to post-translational modifications. In fact, recent studies have suggested that the 200 kDa, full-length CDP is proteolytically cleaved during cell cycle progression, generating N-terminal and C-terminal peptides of 90 and 110 kDa, respectively (Fig. 1.3) (Moon et al., 2001). It was found that of the full-length and 110 kDa isoforms, the truncated isoform bound to DNA with greater stability. To the researchers' further surprise, the 110 kDa isoform was found

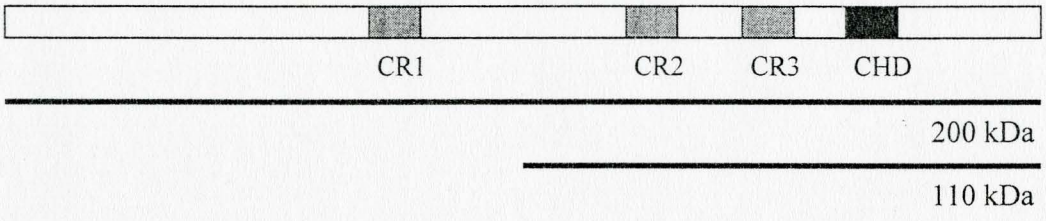


Figure 1.4 Schematic of the CCAAT-displacement protein (CDP). The relative positions of the four DNA binding domains are displayed. The dark lines beneath indicate the lengths of the full-length protein and the C-terminal isoform. CR1 = Cut repeat 1; CR2 = Cut repeat 2; CR3 = Cut repeat 3; CHD = Cut homeodomain.

to behave as a transcriptional activator when co-transfected with a reporter gene construct, while the full-length form upheld its reputation as a repressor. Clearly, CDP/Cut is a complex and incompletely characterized protein, but its apparent importance in the regulation of sialidase expression in mice makes it an interesting target for the modulation of human sialidase expression, with the ultimate goal of activating the sialidase-mediated bypass of Hex A deficiency in human Tay-Sachs patients.

1.6 Alternate Gene Induction as a Potential Therapy for Genetic Disorders

The up-regulation of an alternate endogenous gene product to replace the function lacking due a monogenic disease state has recently become an attractive strategy for therapeutic intervention. Where such an alternate gene product exists, it may be possible to replace proper function while circumventing the numerous problems inherent in typical gene therapy, namely, exogenous gene targeting and delivery, immune response, and longevity of transgene expression. Up until this point, researchers have primarily targeted structural homologues as potential replacements for their non-functioning counterparts. Such is the case with sickle cell disease and beta-thalassaemia, where researchers are seeking to ameliorate the conditions by re-activating fetal haemoglobin (Olivieri & Weatherall, 1998), as well as Duchenne muscular dystrophy, where structural and functional similarities with dystrophin have implicated its homologue utrophin as a therapeutic target (Burton et al., 1999; Krag et al., 2001; Perkins et al., 2001). The discovery that transgenic overexpression of lysosomal sialidase is able to reduce the accumulation of ganglioside GM2 in Tay-Sachs neuroglia cells (Igdoura et al., 1999) provided

evidence that structurally and/or functionally dissimilar proteins may also be targeted for therapeutic up-regulation.

While alternate gene induction offers numerous benefits over conventional gene therapy, a great deal of difficulty exists in identifying effective mechanisms of up-regulation; the mechanism should not only affect up-regulation capable of improving or restoring normal phenotype, but it should ideally be gene and/or tissue-specific. Attempts at up-regulating utrophin to correct Duchenne muscular dystrophy have thus far focussed on two separate promoters responsible for controlling utrophin expression. Studies of these promoters and the elements involved in utrophin expression in muscle, the target tissue for correction of Duchenne muscular dystrophy, have identified a number of potential targets for up-regulation (Perkins et al., 2001). The ultimate goal of this research is to identify the relevant transcriptional regulatory mechanisms operating in muscle, in order to isolate molecular targets for pharmacological intervention. In order to pursue human lysosomal sialidase as a therapeutic alternate gene for Tay-Sachs disease, information about its expression in human neuronal cells, particularly information about its promoter and the regulatory molecules that interact with it, must be thoroughly gathered.

1.7 Research Objectives

As outlined above, lysosomal sialidase acts to bypass β-hexosaminidase A (Hex A) deficiency in Hex A knockout mice. Similarly, overexpression of lysosomal sialidase in human Tay-Sachs neuroglia cells results in the reduction of intralysosomal ganglioside GM2 accumulation. Thus, lysosomal sialidase exists as a potential alternate gene product for the treatment of human GM2 gangliosidosis.

Unfortunately, human lysosomal sialidase is a poorly characterized enzyme and, in particular, little is known about the transcriptional regulatory mechanisms involved in its expression. Consequently, the goals of this research were twofold: (1) to characterize the human lysosomal sialidase promoter, and (2) to identify potential target promoter elements for the up-regulation of lysosomal sialidase in human Tay-Sachs cells and assess the effect of this up-regulation on intralysosomal GM2 accumulation. Because of the previous identification of the CCAAT-displacement protein (CDP) as an important determinant of mouse lysosomal sialidase expression, an emphasis was put on the elucidation of the effects of CDP on the regulation of human lysosomal sialidase expression. Though this research represents the beginning of a long and arduous course of study, the ultimate goal is to identify molecular targets for the eventual pharmacological treatment of the Hex A deficient GM2 gangliosidoses.

CHAPTER 2

Materials and Methods

2.1 Construction of Promoter-Reporter Plasmid

Luciferase reporter plasmids were based upon the pGL3 Basic vector (Promega). To obtain the full-length human lysosomal sialidase promoter vector, two separate PCR reactions on the human sialidase BAC (NCBI accession # AF134726) were performed. One reaction (forward primer ACTGAGCCGTTCAAGCATTT, reverse primer GCGACCCTGGCAGCTAGA) amplified 1.8 kb immediately upstream of the transcriptional start, while the other reaction (forward primer GAGTGCAGAACCCTGTAGCC, reverse primer TCCTGACCTCAGGGTGATCT) amplified 1.2 kb further upstream of the first fragment, resulting in the amplification of ~ 2.9 kb of total promoter sequence. The primer sets were designed such that both fragments would contain a *BsmI* site for later religation (Fig 2.1). The products of both reactions were then subcloned into the TA-cloning site of pCR2.1 (Invitrogen) and their orientations confirmed by restriction digest. The 1.2 kb promoter fragment/pCR2.1 construct was digested with *EcoRV* and the 1.8 kb promoter fragment/pCR2.1 construct was digested with *BamHI*; both digests purified using the Geneclean II kit (Q-BIOgene), and the *BamHI* overhang on the 1.8 kb/pCR2.1 fragment was blunted using the Klenow fragment. Both constructs were then further digested with *BsmI* and run on a 1% agarose gel; a ~ 5 kb band consisting of the 1.2 kb promoter fragment and the pCR2.1 backbone was isolated, as was a 1.8 kb band representing the 1.8 kb promoter fragment. The 1.8 kb promoter fragment was ligated into the 1.3 kb/pCR2.1 backbone by blunted *BamHI/EcoRV* and cohesive *BsmI* overhangs, resulting in ~ 2.9 kb of sialidase promoter sequence cloned in pCR2.1. The full-length promoter sequence was then removed from pCR2.1 by digest with *KpnI* and *XhoI* and cloned into pGL3 Basic by cohesive ligation. Positive clones were

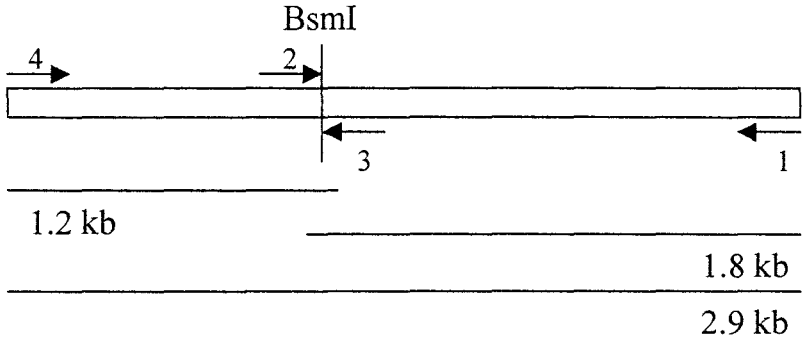


Figure 2.1 Schematic of Primer System for PCR of Human Sialidase Promoter Sequence. Primers denoted 1 and 2 amplified 1.8 kb of promoter sequence adjacent to the transcriptional start, while primers denoted 3 and 4 amplified 1.2 kb further upstream. Primers were designed such that both fragments contained the *BsmI* restriction site.

identified by restriction digest and the promoter sequence was confirmed by sequencing.

2.2 Construction of Promoter Deletion-Reporter Plasmids

Promoter deletion-reporter constructs were prepared from pGL3/fullprom with exonuclease III according to New England BioLabs Exo-Size™ Deletion protocol. This protocol yielded four constructs, named for the approximate length of promoter sequence remaining – del2kb, del1.8kb, del1.5kb, and del1kb. A construct lacking the putative binding site for CDP (del2kb-CDP) was created by digesting the del2kb construct with *NsiI*, which removed a ~ 60 bp fragment containing the binding site. Though not visible by restriction digest, sequencing confirmed the loss of the CDP binding site from this construct. Two additional deletion constructs were created using enzymatic strategies. Digestion of pGL3/fullprom with the blunt-cutting enzymes *MslI* and *Ecl136II* followed by religation resulted in a deletion with ~ 700 bp of promoter sequence remaining (del700bp). Similarly, digestion of pGL3/fullprom with *PvuII* and *Ecl136II* followed by religation resulted in a deletion construct consisting of ~ 100 bp of promoter sequence (del100bp) (Fig. 2.2).

2.3 Cell Culture and Transient Transfection Assays

Normal and Tay-Sachs neuroglia cells (CRB/Nor and CRB/TSD, respectively) were the generous gifts of Dr. Brooks (Kingsbrook Jewish Medical Center, NewYork, NY). 293 cells were maintained in F-11 medium supplemented with 10% horse serum, penicillin + streptomycin, and fungizone. All other cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf

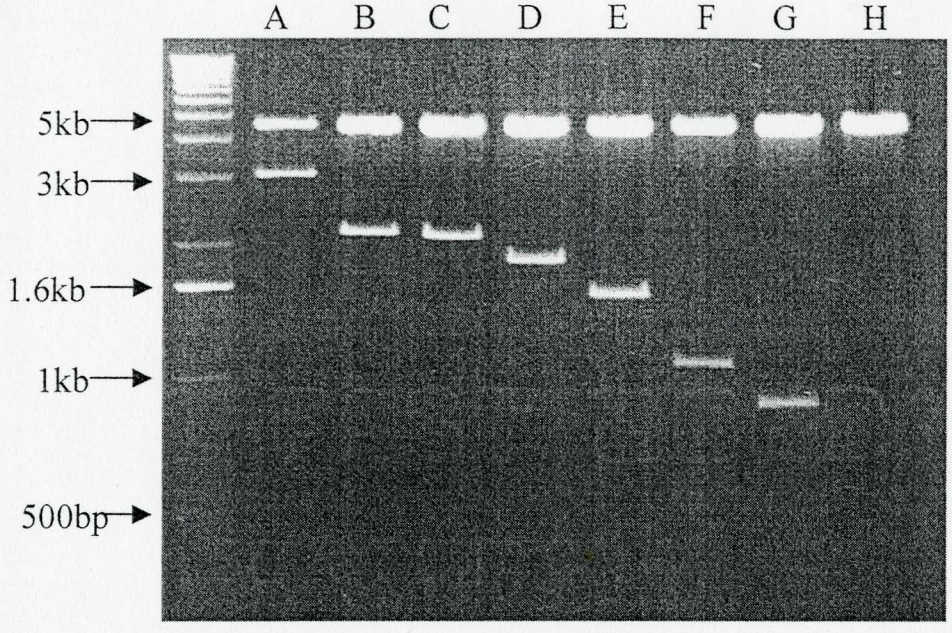


Figure 2.2 Analysis of human lysosomal sialidase promoter deletion constructs by restriction digest with *NotI*. Digestion of the promoter deletion constructs with *NotI* liberates the promoter insert from the pGL3 backbone. In each lane, the highest band represents the pGL3 backbone while the lower band represents the length of promoter sequence present in each construct. A = fullprom; B = del2kb; C = del2kb-CDP; D = del1.8kb; E = del1.5kb; F = del1kb; G = del700bp; H = del100bp (the 100 bp insert was visible on the gel, but was difficult to photograph).

serum, penicillin + streptomycin, and fungizone. Transfections were performed with the Superfect Transfection Reagent (Qiagen). Cells in 24-well plates at approximately 40-60% confluency were transfected with 0.5 μg of reporter plasmid, 0.25 μg of effector plasmid, 67 ng pRL-CMV, and 1.2 μl of Superfect per well. Though they received the same amount of DNA, it was determined that the primary cell lines, CRB/Nor and CRB/TSD, required 2.5 μl of Superfect per well for maximum transfection efficiency (see section 4.1). Cells were incubated for 72 hrs post-transfection, at which time firefly and renilla luciferase activities were measured using the Dual Luciferase Assay (Promega). Unless otherwise noted, results were normalized against renilla activity and data was presented with error bars representing standard deviation (n = 3).

2.4 RNA Extraction and Reverse-Transcriptase PCR (RT-PCR)

CRB/Nor and CRB/TSD cells were cultured to high confluency in 75 mm^2 dishes. RNA was extracted from the cells using Trizol ® reagent (Gibco) according to the manufacturer's instructions. The resulting RNA pellet was dissolved in DEPC-dH_20.

RNA to be used in cDNA synthesis was heated to 55°C for 10 min., and then placed on ice. RT-PCR was carried out with 1-5 μg of RNA. Upon completion of the RT-PCR reaction, separate PCR reactions were performed on 3 μl of the RT-PCR mix, one set of reactions using primers designed to amplify a 494 bp fragment of the cDNA for Cux-1 (forward primer TGCTGAAGTGAAAAATCAAGAGG, reverse primer CGGCCAACTCAACTTCTAGG), and the other set using primers designed to

amplify a 289 bp fragment of the cDNA for Cux-2 (forward primer CCGTGCCTGTGTTTGAGG, reverse primer GCCGCCAAAGTGATCTGTA). Each set was composed of reactions performed at different annealing temperatures (55°C, 59°C, and 63°C). Upon completion of the PCR reactions, the resulting samples were run on a 1% agarose gel.

2.5 Construction and Use of Recombinant Adenovirus

Sequence containing luciferase driven by approximately 2 kb of human sialidase promoter was removed from the pGL3/fullprom plasmid described above by digestion with *MslI* and *BamHI*. The resulting fragment was then cloned into the adenovirus (Ad) shuttle plasmid pDC311 by ligation to *StuI* and *BglII* sites. To create the AdCDP$^{831\text{-}1505}$ shuttle, pTri-Ex 2.1/ CDP$^{831\text{-}1505}$ was first digested with *PacI*. The *PacI* overhang was blunted with Mungbean nuclease and the plasmid was further digested with *NotI*, resulting in the removal of CDP$^{831\text{-}1505}$ and its associated histidine tag from the pTri-Ex backbone. The CDP$^{831\text{-}1505}$ fragment was then cloned into the Ad shuttle plasmid pDC316 by ligation into *SmaI* and *NotI* sites. To create the AdCDP$^{1\text{-}1109}$ shuttle, the pXM/CDP$^{1\text{-}1109}$ expression construct was first digested with *XhoI*. The *XhoI* overhang was blunted with Mungbean nuclease and the plasmid was further digested with *NotI* – this resulted in the removal of the CDP$^{1\text{-}1109}$ sequence along with its associated HA-tag from the pXM backbone. The CDP$^{1\text{-}1109}$ fragment was then cloned into pDC316 by cohesive ligation into *SmaI* and *NotI* sites. In all cases, the recombinant shuttle and the Ad genomic plasmid pBHGloxΔE1,3Cre were co-transfected into 293 cells using the calcium phosphate precipitation technique as

previously described (Ng et al., 2000). Plaques were picked and used to infect 293 cells in order to obtain a high titre viral preparation. Cells were infected with serial dilutions of the high titre prep. (Note: the human sialidase promoter virus was created, but not used for the purposes of this thesis).

2.6 Antibodies

Rabbit polyclonal antibodies against recombinant human lysosomal sialidase were prepared as described previously (Igdoura, et al, 1998). Other primary antibodies used included human anti-GM2, mouse anti-histidine tag, and mouse anti-HA-tag. Secondary antibodies employed in immunocytochemistry included goat anti-rabbit Texas red conjugated IgG, goat anti-human alexa 488 conjugated IgG, and goat anti-mouse alexa 350 conjugated IgG (Qiagen or Molecular Probes).

2.7 Western Blotting

Cells infected with recombinant adenovirus 72 hrs prior were washed once in PBS and scraped into 500 μl of RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, pH 7.4) and placed in a 1.5 ml microcentrifuge tube. Each tube was then vortexed at high speed for 1 min to ensure complete lysing of the cells, followed by centrifugation at high speed to remove membrane fragments and other particulate matter from the lysates. The protein concentrations of the lysates were measured by Bradford assay (BioRad). An appropriate volume of 5× loading buffer was added to a 50 μl sample of each lysate and the samples were boiled for 10 min. The samples were loaded into the wells of a 12% SDS-polyacrylamide gel with

a 4.5% stacking gel, such that each sample was represented by the same amount of protein, and run at 30 mA for ~ 2 hrs. Gels were blotted onto nitrocellulose membranes. Membranes were blocked for 1 hr at room temperature with 10% nonfat dry milk in TBS (10 mM Tris-Cl, pH 8.0, 150 mM NaCl). The membrane was then incubated with the rabbit-anti-human sialidase antibody at a dilution of 1:100 in 10% milk/TBS overnight at 4°C. The membrane was washed with TBS + 0.05% Tween 20 and then incubated with goat-anti-rabbit IgG conjugated to horseradish peroxidase at a dilution of 1:10,000 for 1 hr at room temperature. The membrane was washed again with TBS + 0.05% Tween 20 and developed using the Western Lightning chemiluminescence reagent (Perkin Elmer) according to manufacturers' instructions. Developed membranes were exposed to Kodak X-OMAT AR scientific imaging film. After this first exposure, membranes were stripped according to the Western Lightning protocol. Stripped membranes were then blocked again with 10% milk in TBS, this time for ½ hr. Following the block, membranes were incubated with the mouse-anti-histidine tag antibody at a dilution of 1:250 overnight at 4°C. Membranes were then washed with TBS + 0.05% Tween 20 and incubated with the secondary antibody, anti-mouse IgG conjugated to horseradish peroxidase, at a dilution of 1:10,000 for 1 hr at room temperature. Following this incubation, membranes were washed, developed, and exposed as described above.

2.8 Assay of Sialidase Enzyme Activity

Cells were washed twice with cold phosphate-buffered saline (PBS) and then scraped in cold PBS. The cells were then centrifuged at high speed for 5 min and the

resulting pellet was resupended in 120 μl of distilled water and lysed by repetitive pipetting. Cell homogenates were assayed for lysosomal sialidase activity using 4-methylumbelliferyl-n-acetyl-α-D-neuramide as a substrate, with incubation for 1 h at 37°C (Potier et al., 1979). Following incubation, reactions were stopped with 2 ml of 0.1M MAP, pH 10.5. Fluorescence was measured on the LS Reader Plate Fluorometer (Perkin Elmer). Protein concentrations of the cell lysates were measured using Bradford protein assay (BioRad). Data was presented with error bars representing standard deviation (n = 3).

2.9 Immunocytochemistry

Treated cells, grown on coverslips in 24-well plates, were washed in phosphate-buffered saline (PBS) and fixed for 30 min in 3.8% paraformaldehyde in PBS. Cells were permeabilized with 0.5% Triton X-100 in PBS for 30 min. After washing twice in cold PBS, cells were blocked with 10% goat serum in PBS for 1 h at room temperature. Primary antibodies were diluted 1:100 (except for anti-GM2, which was diluted 1:200) in 10% goat serum in PBS and incubated with the cells at 4°C overnight. Cells were washed 6 times for 5 min each in PBS containing 0.1% Tween-20. Cells were then incubated with secondary antibodies (diluted 1:500 in 10% goat serum in PBS) for 1 hr at room temperature and washed again 6 times with PBS containing 0.1% Tween-20. A final wash was performed in distilled water to remove residual salts. The coverslips were mounted on slides using Pro-Long antifade solution (Molecular Probes). Antibody labelling experiments were analyzed

on a Zeiss Axiovert 200 inverted light microscope (Carl Zeiss). Images were obtained with 40×/0.75 plan-NEOFLUA (Zeiss) objective.

2.10 Ganglioside Labelling, Extraction and Thin Layer Chromatography (TLC)

From the time of their plating into 100 mm dishes, cells were maintained in DMEM and 1 μCi/μl L-[3-^{3}H] serine (Amersham) at a concentration of 0.71 μCi per dish. Cells were allowed to grow in this media for 72 hrs, after which time they were infected with recombinant adenovirus. Cells were scraped and pelleted 72 hrs post-infection.

Cell pellets were resuspended in 1 ml of chloroform:methanol:dH_20 (30:60:8) and probe sonicated for 30 sec. Homogenates were then centrifuged at high speed for 5 min and the supernatant was collected. Cell pellets were extracted a total of three times, and the supernatants were pooled. KOH was then added to each sample up to pH 11, and the samples were put in a 50°C water bath for 4 hrs. The pH was checked periodically during the incubation period and readjusted to 11 if necessary. After incubation, the samples were cooled on ice and the pH was adjusted to 7 with HCl. Samples were centrifuged at high speed for 5 min to remove any residual particulate matter, then the fluid supernatants were dialyzed in Spectra/Por tubing (MWCO - 6-8,000) against 5 L of ddH_20 at 4°C for 48 hrs (ddH_20 changed ×2). After dialysis, the samples were placed in pyrex tubes and frozen. The frozen samples were then lyophilized until dry, and the resulting powder was dissolved in the minimum volume of chloroform:methanol:dH_20 (10:10:1). Dissolved samples were spotted onto a TLC plate at 1 cm intervals and the plate was run in pure acetone, dried, then run in

chloroform:methanol:0.2% $CaCl_2$ (60:35:8). After TLC, the plate was dried and exposed to Hyperfilm-βmax autoradiographic film (Amersham) in an Amersham hypercassette for 7 days. After exposure, gangliosides were stained by spraying TLC plates with Resorcinol/HCl solution, allowing them to dry, and heating them in an oven at 110°C for 20 min.

CHAPTER 3

Results

3.1 Determination of ideal transfection conditions for CRB/Nor and CRB/TSD cells.

The normal and Tay-Sachs cells (CRB/Nor and CRB/TSD, respectively) obtained from Dr. Brooks at the Kingsbrook Jewish Medical Centre in New York were untransformed, primary neuroglia cells. Because of the inherent difficulties that exist in the efficient transfection of primary cell lines, it was necessary to assess various transfection conditions with the CRB cells in order to determine how to achieve maximum transfection efficiency in future experiments. This assessment was carried out by transfecting CRB/Nor cells with various amounts of a constitutively active promoter-reporter plasmid, pGL3-PGK, and the transfection reagent Superfect. The various amounts of reagents were also delivered in various ratios. The different combinations of DNA and Superfect assessed were – (1) 0.5 μg DNA, 2.5 μl Superfect; (2) 1 μg DNA, 5 μl Superfect; (3) 1.5 μg DNA, 7.5 μl Superfect; (4) 0.5 μg DNA, 1 μl Superfect; (5) 1 μg DNA, 2 μl Superfect; and (6) 1.5 μg DNA, 3 μl Superfect. The transfections were performed on CRB/Nor cells in triplicate in a 24-well plate. Luciferase assay of the cell lysates 72 hrs post-transfection revealed the 0.5 μg DNA, 2.5 μl Superfect (a 1:5 ratio) combination as the most consistent and efficient transfection condition (data not shown). This combination of DNA and Superfect was subsequently used for all transfection experiments with the primary cell lines CRB/Nor and CRB/TSD.

3.2 *In vitro* comparison of human and mouse lysosomal sialidase promoter activities.

When the sialidase bypass was first discovered in Hex A knockout mice, there was debate as to whether the bypass arose due to a higher affinity of the mouse enzyme for GM2 or rather to higher levels of the enzyme in ganglioside-producing tissues. The observation that overexpression of lysosomal sialidase in human Tay-Sachs cells results in the depletion of accumulated ganglioside GM2 in a manner similar to the bypass in Hex A knockout mice (Igdoura et al., 1999) has led to the belief that the difference between human and mouse sialidases is a matter of quantity rather than quality. In order to determine if the difference in enzyme levels between mice and humans is due to differences in transcriptional regulation, we sought to compare the basal activities of the human and mouse lysosomal sialidase promoters in both human and mouse cells. This was done by transfecting the human cell line CRB/Nor and the mouse cell line TIB73 with similar length human and mouse promoter-reporter constructs and measuring their respective activities by dual luciferase assay (as mentioned previously, the human promoter-reporter construct contained ~ 2.9 kb of regulatory sequence, while the corresponding mouse construct contained ~ 3.5 kb of sequence). A similar pattern of expression was observed in both human and mouse cell lines, with the mouse promoter displaying 6-7-fold higher activity than the human promoter (Figs. 3.1 and 3.2). These *in vitro* results suggest that the higher amounts of lysosomal sialidase responsible for the sialidase-mediated bypass of Hex A deficiency in mice may be at least partially due to a more active promoter. They do not, however, rule out the possibility of post-translational phenomena also having an influence on the amount of functional lysosomal sialidase

in the cell. It is also possible that the extra 600 bp of sequence in the mouse promoter construct contains enhancer sequences that result in its higher activity.

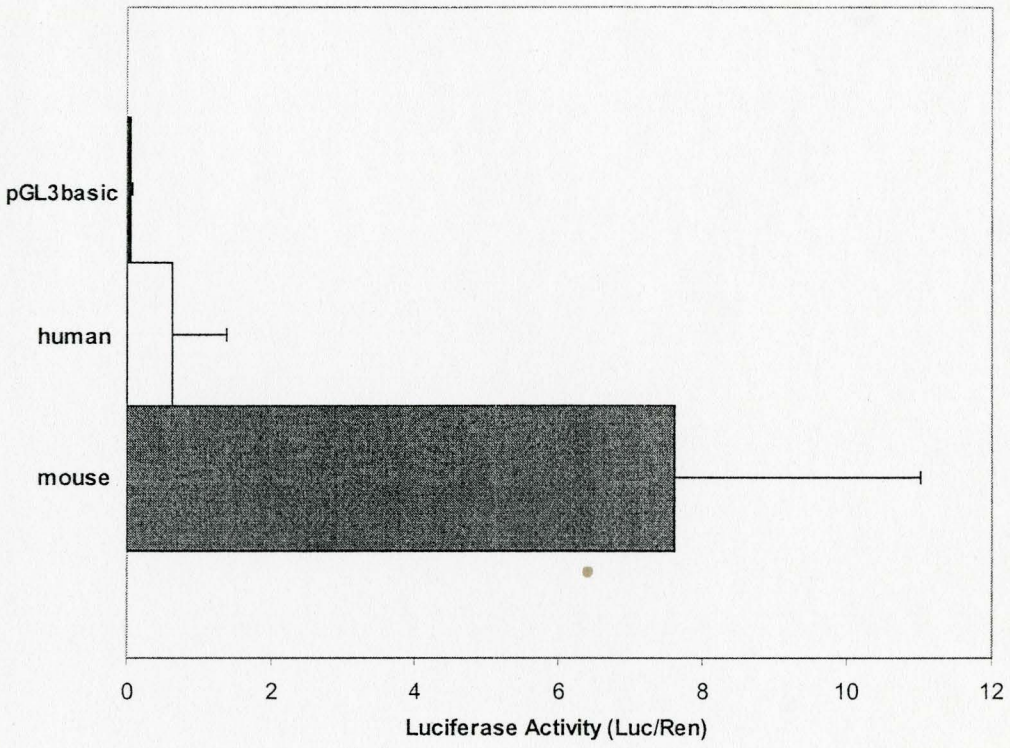


Figure 3.1 Comparison of human and mouse lysosomal sialidase promoter activities in mouse liver cells. TIB73 cells were transfected with pGL3basic and pGL3 constructs containing either the human or mouse lysosomal sialidase promoter. The murine promoter construct showed approximately 7-fold higher luciferase activity than the human construct.

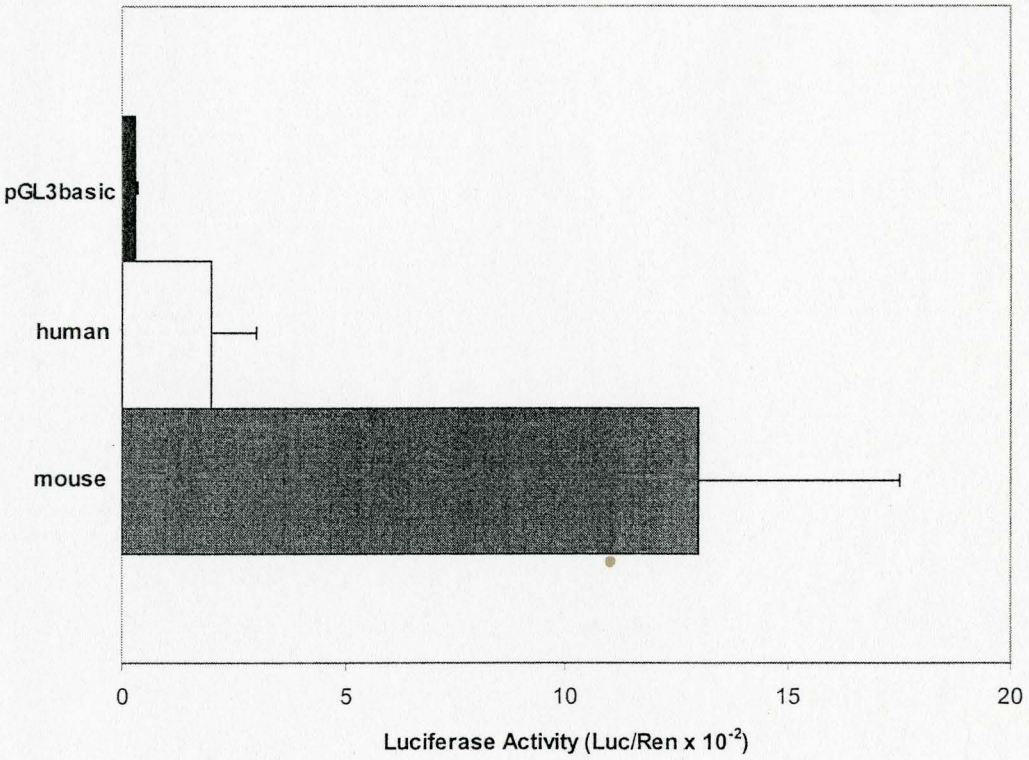


Figure 3.2 Comparison of human and mouse lysosomal sialidase promoter activities in human neuroglia cells. CRB/Nor cells were transfected with pGL3basic and pGL3 promoter-reporter constructs containing either the human or mouse lysosomal sialidase promoter. The murine promoter showed approximately 6-fold higher relative luciferase activity than the human promoter.

3.3 *In vitro* activity of human lysosomal sialidase promoter deletions.

In order to identify the key regulatory regions of the human sialidase promoter, as well as the minimal promoter sequence necessary for transcription of the gene, we generated a series of promoter deletion-reporter constructs and assayed for their activity in various cell lines. Eight constructs were created, each named for the approximate length of promoter sequence of which they were composed: (1) fullprom – contained the full-length promoter sequence, approximately 2.9 kb; (2) del2kb – contained ~ 2 kb of promoter sequence; (3) del2kb-CDP – contained ~ 2 kb of promoter sequence and was lacking the putative binding site for human CDP; (4) del1.8kb – contained ~ 1.8 kb of promoter sequence; (5) del1.5kb – contained ~ 1.5 kb of promoter sequence; (6) del1kb – contained ~ 1 kb of promoter sequence; (7) del700bp – contained ~ 700 bp of promoter sequence; and (8) del100bp – contained ~ 100 bp of promoter sequence. Three cell lines (CRB/Nor, TIB73, and 293) were transfected with these constructs in triplicate in 24-well plates and subsequently assayed for luciferase activity. The promoter deletions displayed a similar pattern of activity in all three cell lines (Figs. 3.3-3.5), suggesting that the regulatory machinery involved in human lysosomal sialidase expression is not cell-type specific, and that it may also be conserved among mammals. The difference in activity observed between fullprom and del2kb in all three cell types indicates the presence one or more important silencer elements between 2-3 kb of the promoter sequence. Likewise, the marked reduction in luciferase activity observed with del1kb indicates the presence of an enhancer between 1-1.5 kb of the promoter, as well as another important silencer element between 700 bp-1 kb. Essentially no activity is observed with del100bp in all cell types, implying that the elements necessary for minimal transcription of the

sialidase gene exist between 100 bp to 700 bp upstream of the transcriptional start site. It is interesting to note that del2kb-CDP showed greater activity than del2kb in only one cell type (293), and even then the increase was only slight (~ 50%). This observation was unexpected, as it opposed the results of similar experiments with the mouse promoter in which a dramatic increase in promoter activity was associated with the loss of the putative binding site for CDP. The results observed with del2kb-CDP suggest that CDP does not play a major role in the repression of human lysosomal sialidase expression by the interaction with the putative binding site at this location. It remains possible, however, that CDP binds to the promoter at different sites, or that it may have a regulatory role in sialidase expression other than that of a repressor.

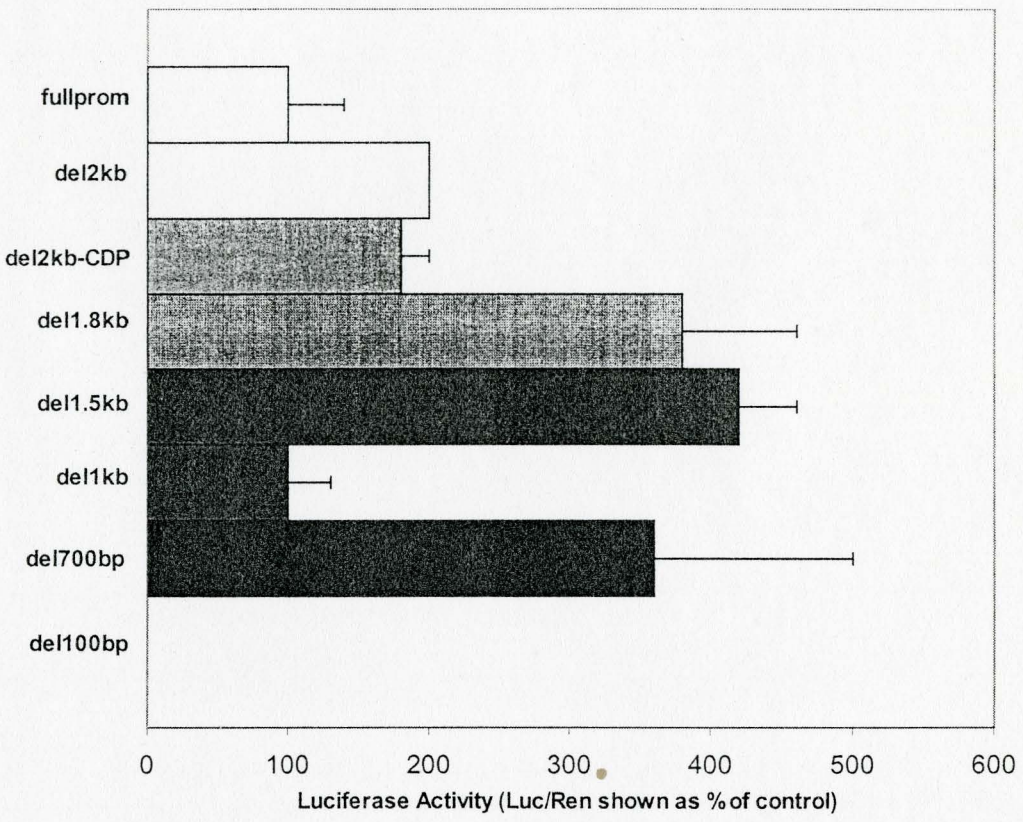


Figure 3.3 Activity of human lysosomal sialidase promoter deletions in human neuroglia cells. The dramatic increase in activity observed when the full length (2.9 kb) promoter is reduced to ~ 2 kb suggests the presence of an important silencer element in the region between 2 kb and 2.9 kb upstream of the transcriptional start site. Similarly, the low activity of del1kb suggests the presence of an enhancer between 1-1.5 kb, and the high activity of del700bp suggests the presence of another important silencer between 700bp-1kb. The activity of the 2.9 kb full length promoter-reporter construct is considered 100%.

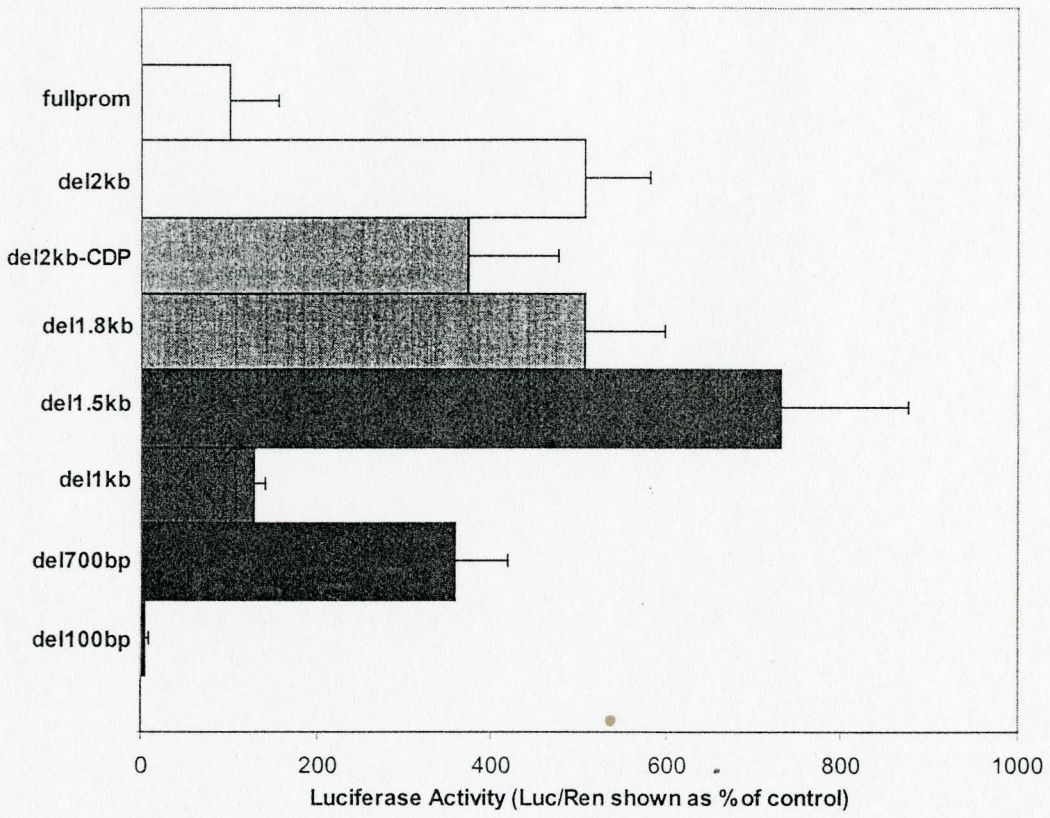


Figure 3.4 Activity of human lysosomal sialidase promoter deletions in mouse liver cells. The pattern of promoter deletion activity in mouse liver cells was similar to that observed in human neuroglia cells, suggesting that the transcription factors responsible for human lysosomal sialidase gene regulation are not species specific.

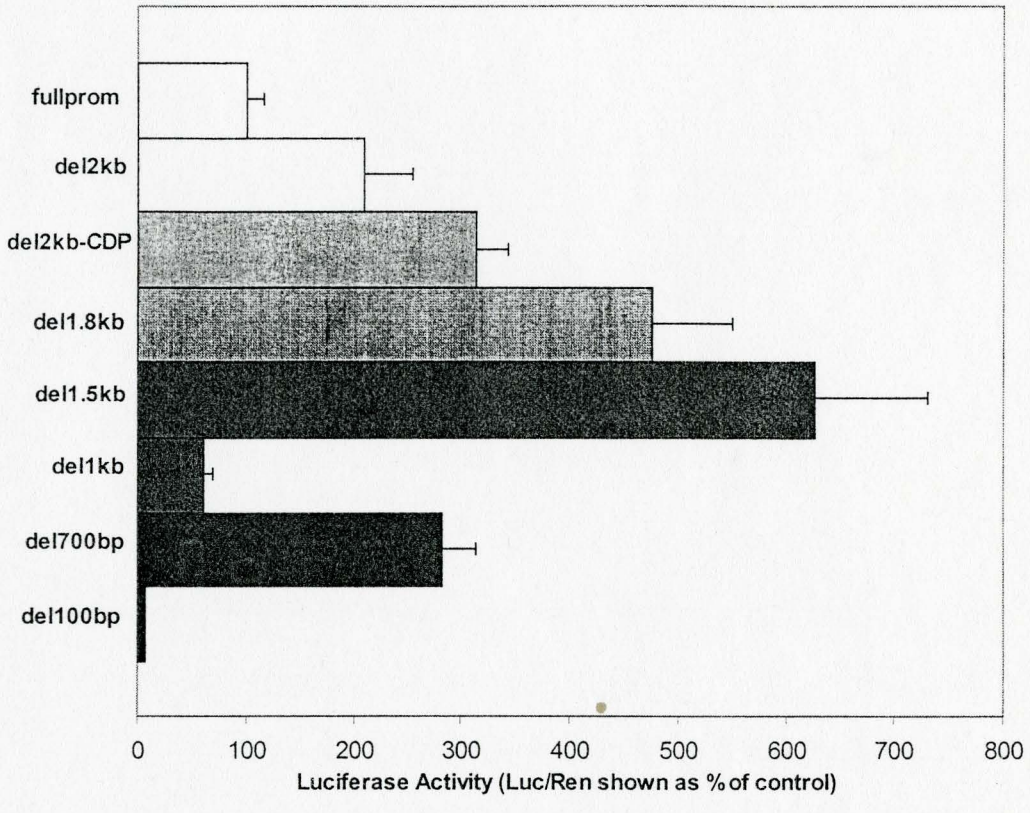


Figure 3.5 Activity of human lysosomal sialidase promoter deletions in human kidney cells. The pattern of promoter deletion activity in human kidney cells (293 cells) was similar to that observed in both human neuroglia cells and mouse liver cells, suggesting that the regulatory machinery responsible for human lysosomal sialidase gene expression might not cell-type specific.

3.4 Human lysosomal sialidase promoter modulation by MyoD, NeuroD1, NeuroD2, and NeuroD3.

In order to identify transcription factors that may participate in the regulation of the human lysosomal sialidase gene, the promoter sequence was analyzed for putative transcription factor binding sites. Analysis of the human lysosomal sialidase promoter with MatInspector 2.2 revealed the presence of multiple potential E-boxes, short nucleotide sequences to which MyoD and NeuroD transcription factors can bind. The NeuroD factors, NeuroD1, NeuroD2, and NeuroD3, are known for their roles in the regulation of neuronal gene expression, while experiments with the mouse lysosomal sialidase promoter have revealed the ability of MyoD to upregulate promoter activity *in vitro* (Champigny, unpublished data). For these reasons, we chose to analyze the effect of these factors on the activity of the full-length promoter-reporter construct. Mammalian expression vectors containing the cDNAs of MyoD, NeuroD1, NeuroD2, and NeuroD3 were co-transfected with the full-length promoter-reporter construct in triplicate into CRB/Nor cells in a 24-well plate. Cell lysates were collected 72 hrs post-transfection and analyzed by dual luciferase assay. The results of the luciferase assay showed no clear modulation of promoter activity in human neuroglia cells by any of the factors tested (Fig. 3.6).

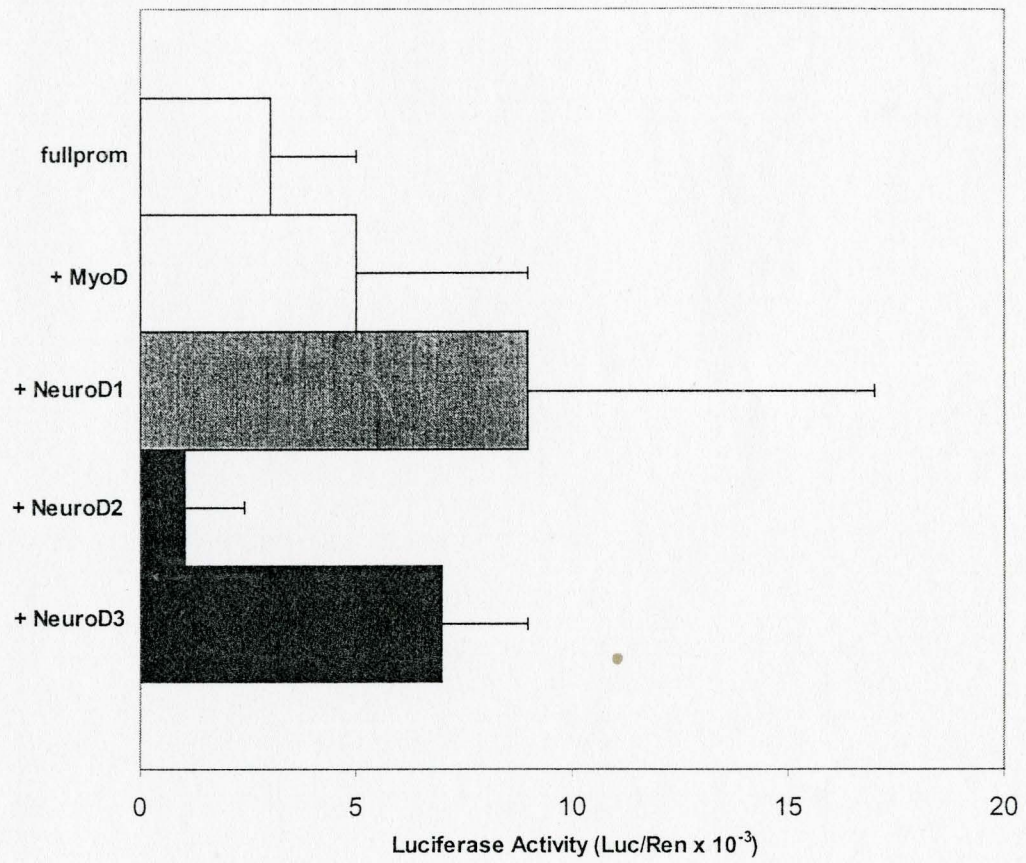


Figure 3.6 Modulation of the human lysosomal sialidase promoter by the transcription factors MyoD, NeuroD1, NeuroD2, and NeuroD3. No clear modulation of promoter activity was observed upon co-transfection with these factors.

3.5 Human lysosomal sialidase promoter modulation by various truncations of the transcription factor CDP.

As discussed previously, the removal of the putative binding site for CDP from the mouse lysosomal sialidase promoter results in a dramatic increase in promoter activity (Champigny, unpublished results). Though removal of the CDP binding site from the human lysosomal sialidase promoter did not result in an increase in activity, the mere presence of the site as well as numerous CCAAT boxes in the human promoter suggests that CDP or isoforms thereof play some kind of role in lysosomal sialidase expression. In order to test this hypothesis, we first obtained six CDP mammalian expression constructs, each containing a unique region of the human CDP cDNA, from Dr. Alain Nepveu at McGill University in Montreal (Fig. 3.7). We then co-transfected each of the CDP constructs with the human lysosomal sialidase promoter-reporter construct in triplicate into CRB/Nor cells in a 24-well plate. Cell lysates were collected 72 hrs post-transfection and subsequently analyzed for luciferase activity by dual luciferase assay. Because of the different combinations of the CDP DNA-binding domains encoded by the various constructs, we hypothesized that each would have a unique effect on lysosomal sialidase promoter activity *in vitro*. This hypothesis was confirmed by the results of the first experiment, where a wide range of promoter modulation was observed (Fig. 3.8). The slight increase in promoter activity upon co-transfection with the CDP^{full} was unexpected, as it is described in the literature primarily as a transcriptional repressor. However, CDP has been known to act as a transcriptional activator in the context of certain promoters. This may be the case with the human lysosomal sialdiase promoter, possibly due to a unique mechanism of DNA binding or the interaction with other transcription factors.

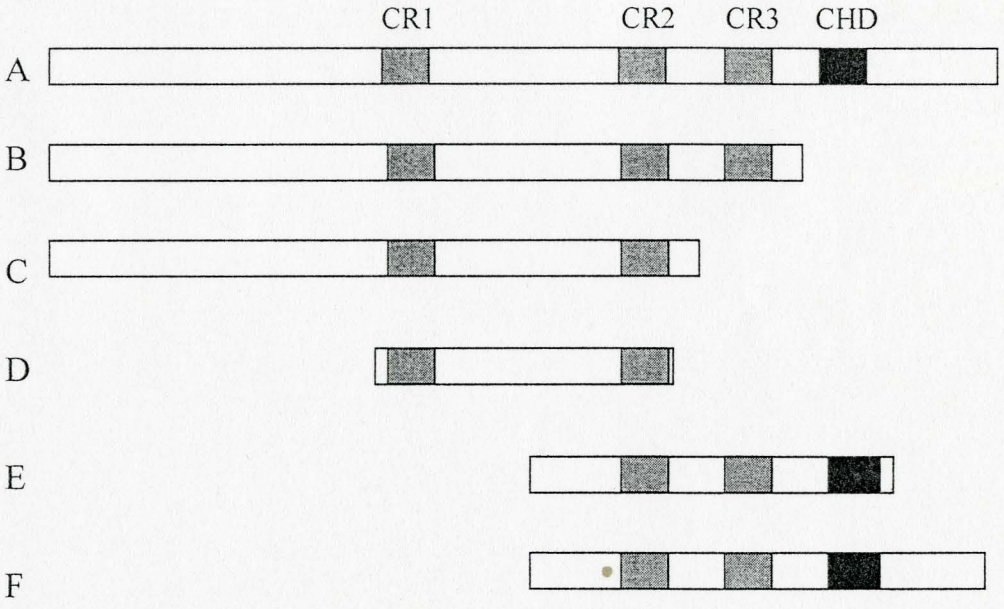


Figure 3.7 Schematic of CDP truncations. The six constructs each contained a unique region of the human CDP cDNA, and thus encoded a unique combination of the CDP DNA-binding domains. Each construct is designated by the amino acid sequence it encodes. A = full-length CDP, CDP^{full}; B = CDP^{1-1308}; C = CDP^{1-1109}; D = $CDP^{522-1027}$; E = $CDP^{831-1336}$; F = $CDP^{831-1505}$.

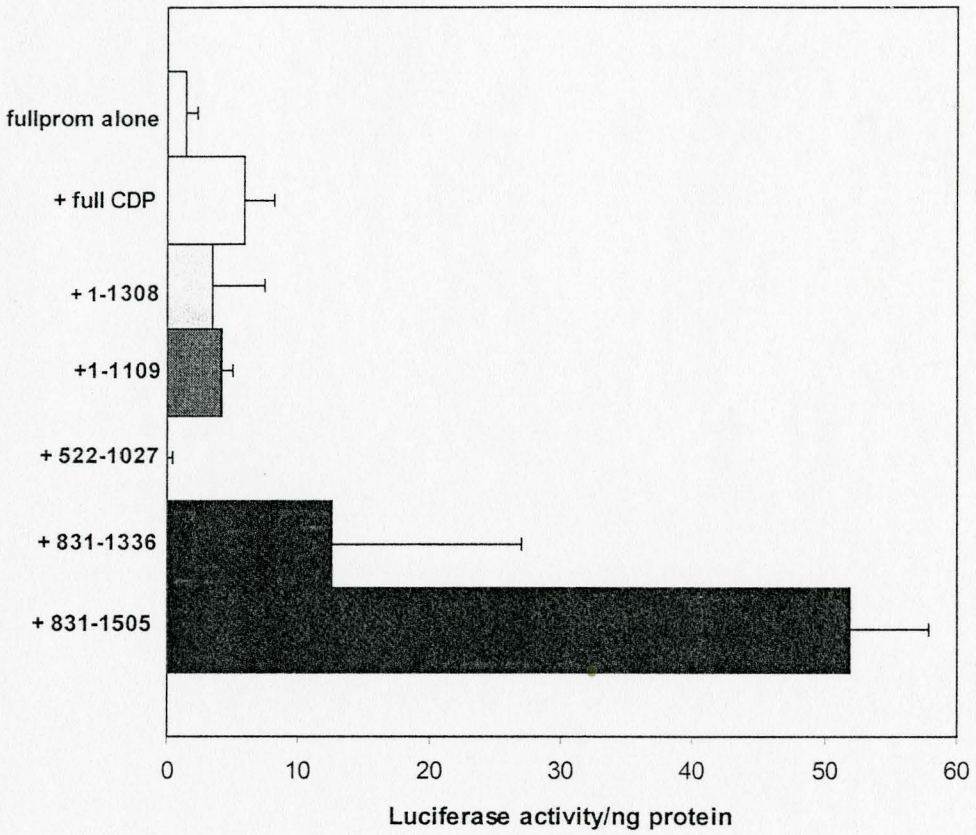


Figure 3.8 Modulation of the human lysosomal sialidase promoter by truncations of the transcription factor CDP. A significant (~ 50-fold) increase in promoter activity was observed upon co-transfection with $CDP^{831-1505}$. Activity is expressed as Luciferase activity/ng protein.

Co-transfection of the promoter-reporter construct with $CDP^{831-1505}$ – it caused an apparent 50-fold increase in promoter activity. Because of highly variable renilla luciferase measurements, luciferase activity in this experiment had to be normalized against protein concentration. The variability in renilla data was observed between the treatments with the different CDP truncations, while the measurements remained relatively consistent within the triplicates of each treatment. This suggests that the different CDP truncations may have each had a unique effect on the CMV promoter regulating renilla expression. Thus, in order to confirm the results of this first experiment, the same co-transfection experiment was repeated three more times – a similar pattern of promoter activity was observed in each experiment (Fig. 3.9). The consistency of the luciferase assay results obtained from these transfection experiments confirmed the ability of the CDP to regulate human lysosomal sialidase promoter activity.

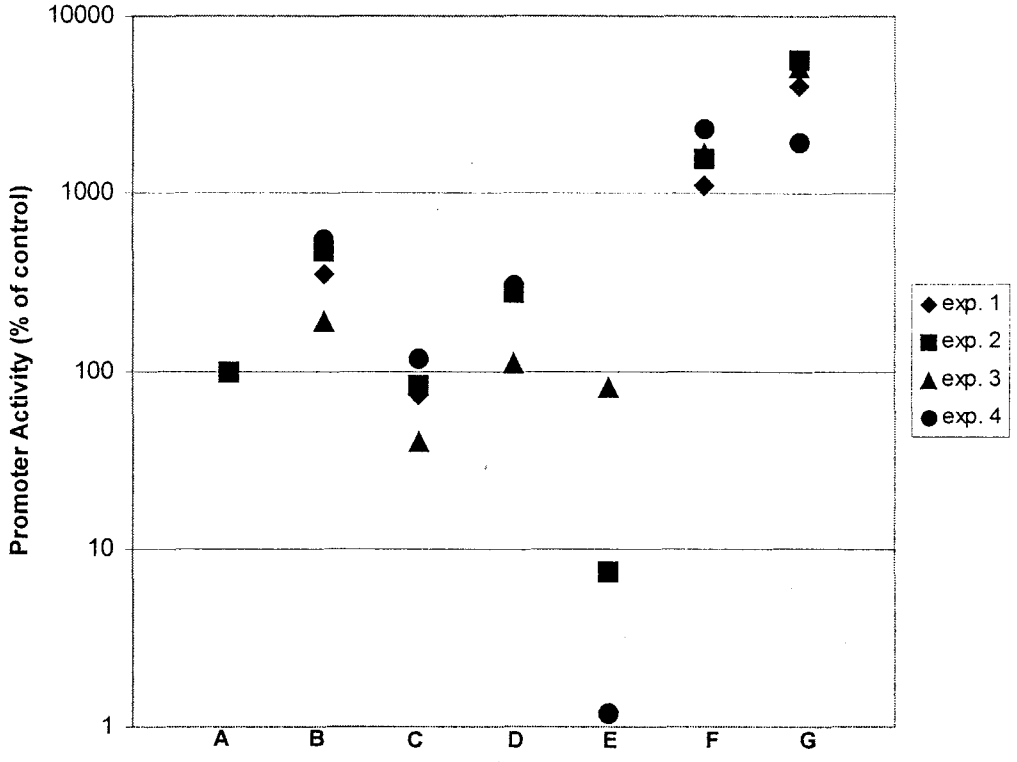


Figure 3.9 Modulation of human lysosomal sialidase promoter activity by CDP truncations over four experiments. The pattern of promoter modulation by the CDP truncations was consistent over four separate co-transfection experiments. In particular, the dramatic increase caused by $CDP^{831\text{-}1505}$ was confirmed. A = human promoter-reporter construct alone, luciferase activity normalized to 100%; B = co-transfection with CDP^{full}; C = co-transfection with $CDP^{1\text{-}1308}$; D = co-transfection with $CDP^{1\text{-}1109}$; E = co-transfection with $CDP^{522\text{-}1027}$; F = co-transfection with $CDP^{831\text{-}1336}$; G = co-transfection with $CDP^{831\text{-}1505}$. The y-axis is displayed as a log scale.

3.6 Confirmation of CDP expression in CRB/Nor and CRB/TSD cells.

If CDP was to be considered an important regulator of lysosomal sialidase expression in neuronal tissue, its expression in neuronal tissue had to be confirmed. We were also interested to find out if the putative neuronal-specific CDP homologue, Cux-2, was present in the neuronal regulatory environment. For these reasons, total RNA was extracted from both CRB/Nor and CRB/TSD cells; this RNA was then used in reverse-transcriptase PCR (RT-PCR) reactions, followed by PCR using primers specific for human CDP cDNA or human Cux-2 cDNA. The results of these reactions confirmed the expression of CDP in both CRB/Nor and CRB/TSD cell lines, while indicating a lack of Cux-2 expression (Figs. 3.10 and 3.11). These results are in accordance with the suspected importance of CDP in the regulation of lysosomal sialidase expression in neuronal tissue, however, the CRB/Nor and CRB/TSD cell lines are of embryonic origin and thus are not terminally differentiated; the persistence of CDP expression upon terminal differentiation can therefore not be assumed. Likewise, the apparent lack of Cux-2 expression in CRB/Nor and CRB/TSD cells does not rule out a regulatory role for Cux-2 once the cell differentiates. Nonetheless, these results provide crucial information about the regulatory environment of our model systems for normal and Tay-Sachs primary neuronal cells.

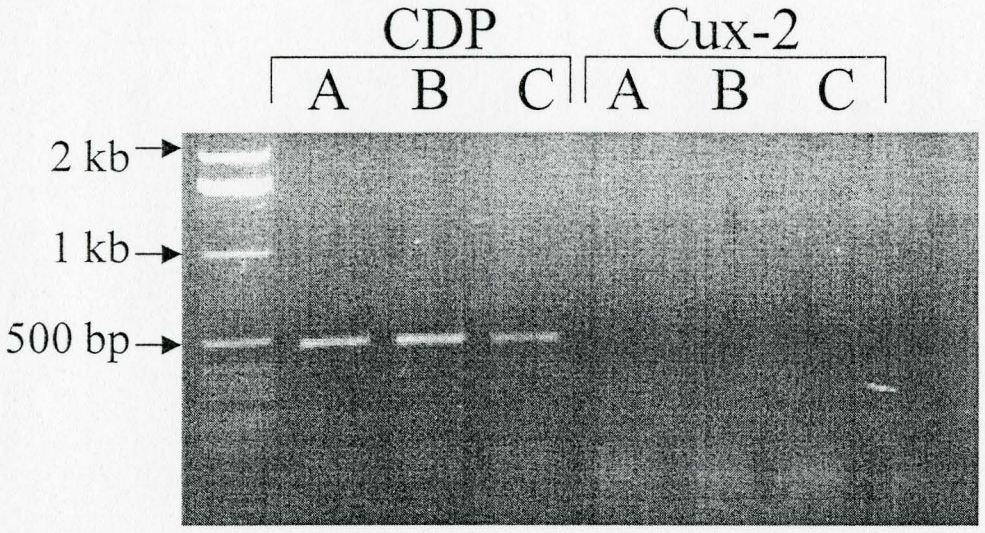


Figure 3.10 Confirmation of CDP expression in CRB/Nor cells. Total RNA was extracted from CRB/Nor cells and subjected to RT-PCR. The resulting cDNA was used in PCR reactions with primers designed to amplify a 494 bp fragment of the cDNA for CDP or a 289 bp fragment of the cDNA for Cux-2, a neuronal specific CDP homologue. The 494 bp fragment was successfully amplified, indicating the expression of CDP in CRB/Nor cells, but the 289 bp band was not amplified, suggesting that Cux-2 is not expressed in these cells. PCR reactions were performed at various annealing temperatures. A = 55°C; B = 59°C; C = 63°C.

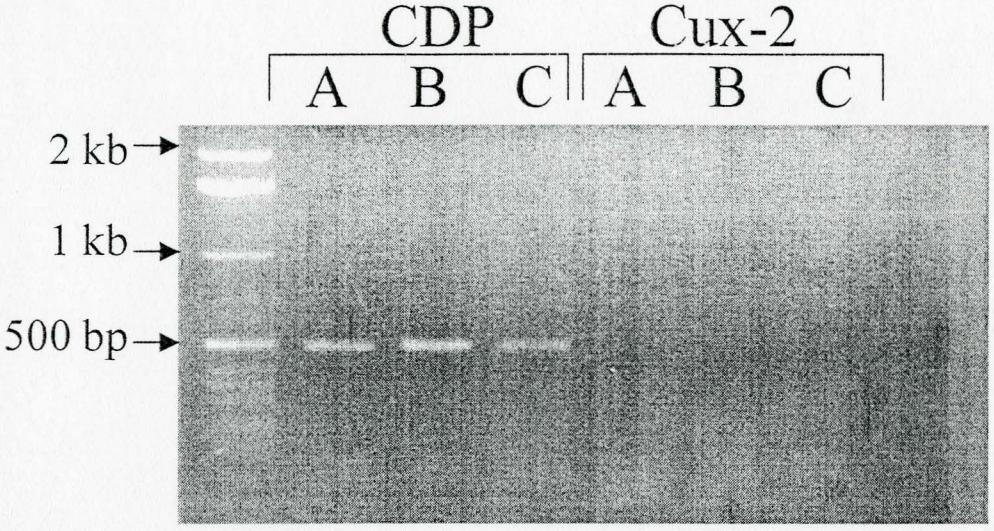


Figure 3.11 Confirmation of CDP expression in CRB/TSD cells. Total RNA was extracted from CRB/TSD cells and subjected to RT-PCR. The resulting cDNA was used in PCR reactions with primers designed to amplify a 494 bp fragment of the cDNA of CDP or a 289 bp fragment of the cDNA of Cux-2, a neuronal specific CDP homologue. As with CRB/Nor cDNA, the 494 bp fragment was successfully amplified and the 289 bp fragment was not, confirming the expression of CDP in CRB/TSD cells and indicating that Cux-2 is not expressed. The PCR reactions were performed using various annealing temperatures. A = 55°C; B = 59°C; C = 63°C.

3.7 Western blot analysis of CRB/Nor and CRB/TSD cells infected with serial dilutions of high titre AdCDP$^{831-1505}$.

The results of the luciferase assays described above clearly demonstrated the ability of the CDP$^{831-1505}$ truncation to up-regulate the human lysosomal sialidase promoter. With this data in mind, we needed to test if the up-regulation in promoter activity caused by CDP$^{831-1505}$ resulted in an increase in sialidase expression *in vitro*. In order to circumvent the low efficiency of gene transfer inherent in typical liposome transfection, we constructed a recombinant adenovirus expressing CDP$^{831-1505}$ with an associated polyhistidine-tag (AdCDP$^{831-1505}$). We infected CRB/Nor and CRB/TSD in 100mm dishes with serial dilutions of a high titre prep of AdCDP$^{831-1505}$ (10^{-2}, 10^{-3}, and 10^{-4}). Cells were harvested 72 hrs post-infection and their lysates were analyzed by Western blot (Fig. 3.12) – equal amounts of protein were loaded in each well, and membranes were probed for both sialidase (44-48 kDa) and CDP$^{831-1505}$ (~ 110 kDa). It was expected that sialidase levels would be increased in cells infected with AdCDP$^{831-1505}$, and that the levels would decrease in a dose-dependent manner as the amount of virus decreased. In both CRB/Nor and CRB/TSD cells, it appears as though the 10^{-2} dilution of AdCDP$^{831-1505}$ succeeded in increasing sialidase levels above those of uninfected cells. In the lanes containing the lysates from CRB/Nor cells infected with the 10^{-3} and 10^{-4} dilutions, however, sialidase levels appear lower than those of uninfected cells. Conversely, in CRB/TSD cells, the 10^{-3} and 10^{-4} dilutions appear to result in sialidase levels greater than those observed with the 10^{-2} dilution. Probing of the 10^{-2} dilution lanes of both cell lines with the mouse anti-his-tag antibody resulted in clear and distinct bands at approximately 110 kDa, indicating

that AdCDP$^{831\text{-}1505}$ was expressing a protein of the correct size with an intact polyhistidine-tag.

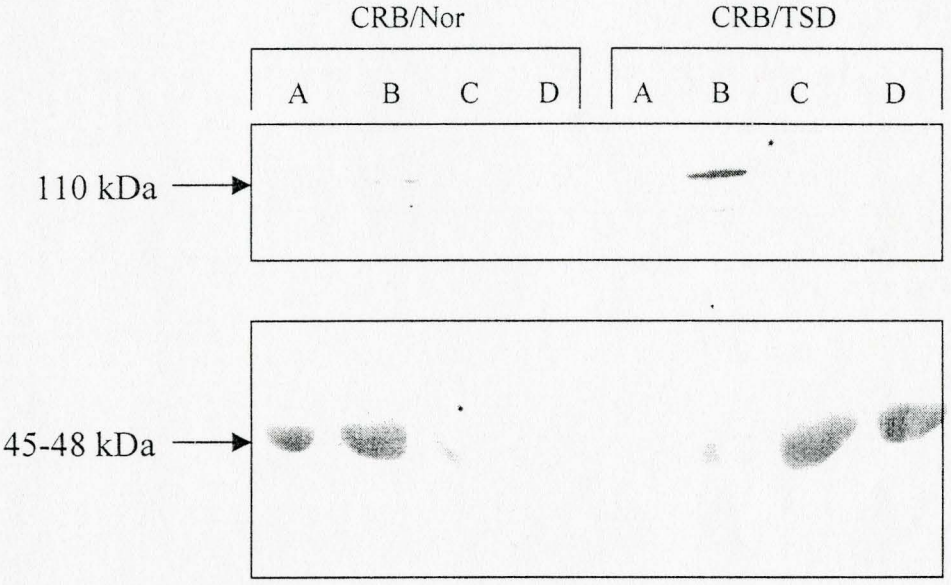


Figure 3.12 Western blot analysis of lysates of CRB/Nor and CRB/TSD cells infected with serial dilutions of high titre AdCDP$^{831\text{-}1505}$. CRB/Nor and CRB/TSD cells were infected with serial dilutions of a high titre prep of AdCDP$^{831\text{-}1505}$, and their lysates were analyzed by Western blot. The membrane was probed sequentially with the anti-human sialidase antibody followed by anti-his-tag antibody – the figure represents an alignment of the two resulting films. The bands at 110 kDa represent the truncated CDP peptide expressed by AdCDP$^{831\text{-}1505}$, while the bands at 44-48 kDa represent human lysosomal sialidase. Lysosomal sialidase levels appear to have increased upon infection with the 10^{-2} dilution, but infection with the 10^{-3} and 10^{-4} dilutions gave conflicting results. A = uninfected; B = 10^{-2} dilution; C = 10^{-3} dilution; D = 10^{-4} dilution.

3.8 Sialidase activity in CRB/TSD and CRB/Nor cells infected with serial dilutions of high titre AdCDP$^{831\text{-}1505}$.

Despite the ambiguous results obtained by Western blot analysis of cells infected with AdCDP$^{831\text{-}1505}$, we were interested to see if the promoter up-regulation caused by CDP$^{831\text{-}1505}$ led to a corresponding increase in sialidase activity. In the assay for sialidase activity, the synthetic substrate 4-methylumbelliferyl-n-acetyl-α-D-neuraminide (Mu-Nana) is cleaved by sialidase, releasing the fluorescent molecule umbelliferone. Because the amount of enzyme activity in a sample is directly related to the amount of functional enzyme present, this fluorometric assay is the most sensitive and specific method available for comparatively quantifying functional lysosomal sialidase. In this case, CRB/Nor and CRB/TSD cells, cultured in six-well plates, were infected in triplicate with serial dilutions of AdCDP$^{831\text{-}1505}$ (10^{-2}, 10^{-3}, and 10^{-4}). Cell lysates were collected 72 hrs post-infection and assayed for lysosomal sialidase activity. It was expected that infection with AdCDP$^{831\text{-}1505}$ would result in an increase in lysosomal sialdiase activity. The results of the sialidase assay confirmed this hypothesis. In both CRB/Nor and CRB/TSD cells, infection with all three dilutions of AdCDP$^{831\text{-}1505}$ resulted in substantial increases in lysosomal sialidase activity (Fig. 3.13). Although infection with the 10^{-2} dilution led to the highest increase in activity (approx. 5-fold), infection with the 10^{-3} and 10^{-4} dilutions also caused significant increases (> 2-fold). These results were unexpected – it was believed that the increase in sialidase activity caused by CDP$^{831\text{-}1505}$ would subside upon dilution of the virus. There exists two explanations for the sustained increases in sialidase activity observed with the 10^{-3} and 10^{-4} dilutions of AdCDP$^{831\text{-}1505}$ in this assay. The first possibility is that CDP$^{831\text{-}1505}$ is capable of significantly up-regulating

the lysosomal sialidase promoter even when present in small amounts. The second, and more likely, possibility is that the high titre preparation of AdCDP$^{831-1505}$ resulted in such a substantial amount of virus that even dilutions of 10^{-3} and 10^{-4} contained enough viral particles to elicit significant lysosomal sialidase promoter up-regulation and a subsequent increase in enzyme activity. The slightly unusual results notwithstanding, it was clear that the increase in human lysosomal sialidase promoter activity observed in the presence of CDP$^{831-1505}$ resulted in a corresponding increase in the level of lysosomal sialidase activity in the cell.

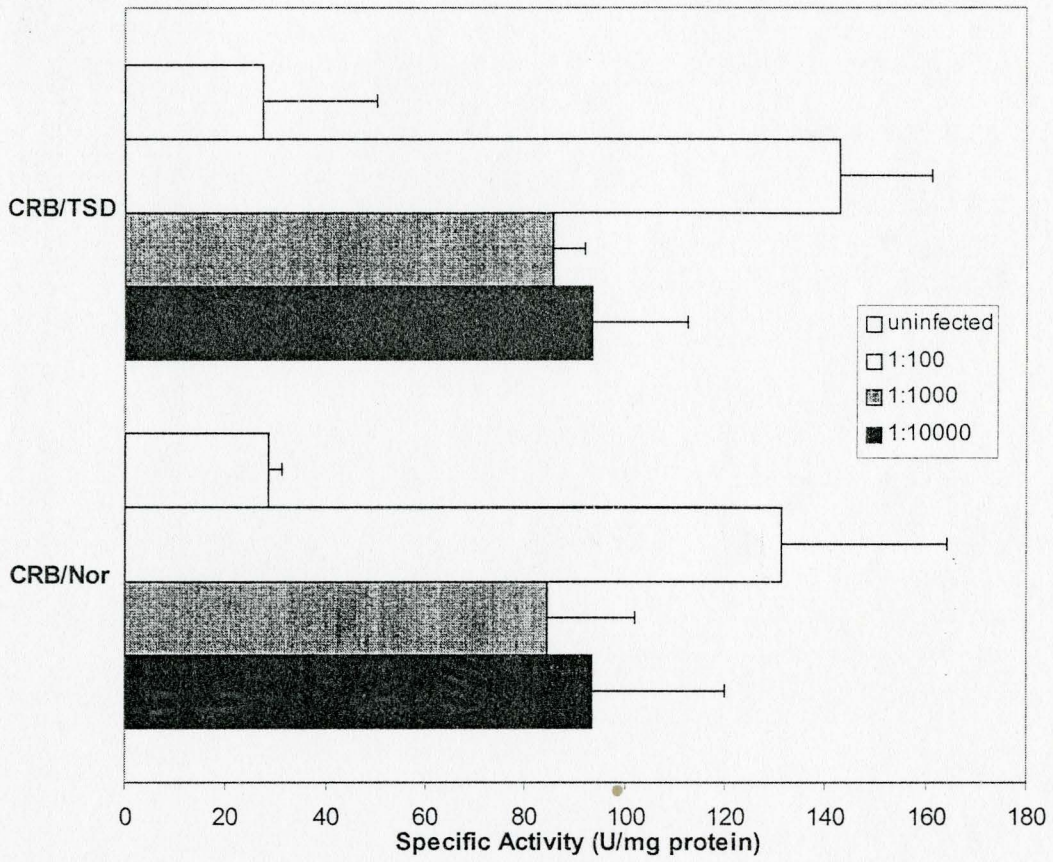


Figure 3.13 Sialidase activity in CRB/TSD and CRB/Nor cells infected with serial dilutions of high titre AdCDP$^{831\text{-}1505}$. Cells were infected with decreasing concentrations of AdCDP$^{831\text{-}1505}$ (10^{-2}, 10^{-3}, and 10^{-4}) and subsequently assayed for sialidase activity. Infection with AdCDP$^{831\text{-}1505}$ resulted in a dramatic increase in sialidase activity in both cell lines. Activity is expressed as U/mg protein, where U = μmol umbelliferone/min.

3.9 Sialidase activity in multiple cell lines infected with AdCDP$^{831\text{-}1505}$.

Because the cells most affected by GM2 gangliosidosis belong to the central nervous system, primary human neuronal cells are the most appropriate model systems for the *in vitro* study of the lysosomal sialidase bypass of Hex A deficiency. The dramatic increase in sialidase activity observed in CRB/Nor and CRB/TSD cells upon infection with AdCDP$^{831\text{-}1505}$ clearly demonstrated the ability of CDP$^{831\text{-}1505}$ to up-regulate sialidase activity in normal primary human neuronal cells and, more importantly, Tay-Sachs primary human neuronal cells. Despite the successful induction of lysosomal sialidase activity in what is considered to be the most appropriate *in vitro* model, we were nonetheless concerned with assessing the tissue and/or cell-type specificity of the CDP$^{831\text{-}1505}$-induced increase in lysosomal sialidase activity. To accomplish this, three human cell lines (CRL 2267, HTB 186, and HELA) and five mouse cell lines (NIH 3T3, C2C12, TIB73, 10T1/2, and MCF7) plated in 6-well plates were either uninfected, infected in duplicate with a 10^{-2} dilution of AdCDP$^{831\text{-}1505}$, or infected in duplicate with a 10^{-2} dilution of Adβ-gal. Lysates were collected 72 hrs post-infection and assayed for sialidase activity. The results of these assays show no significant increase in lysosomal sialidase activity in any of the tested cell lines upon infection with AdCDP$^{831\text{-}1505}$ (Fig. 3.14). Infection with AdCDP$^{831\text{-}1505}$ did appear to induce a slight increase in sialidase activity in HTB 186 cells, but the increase was of a much smaller magnitude than that observed in the CRB cell lines. Though assessment of other primary human cell lines is necessary, these results suggest that the CDP$^{831\text{-}1505}$-induced increase in lysosomal sialidase activity may be restricted to primary human neuronal cells.

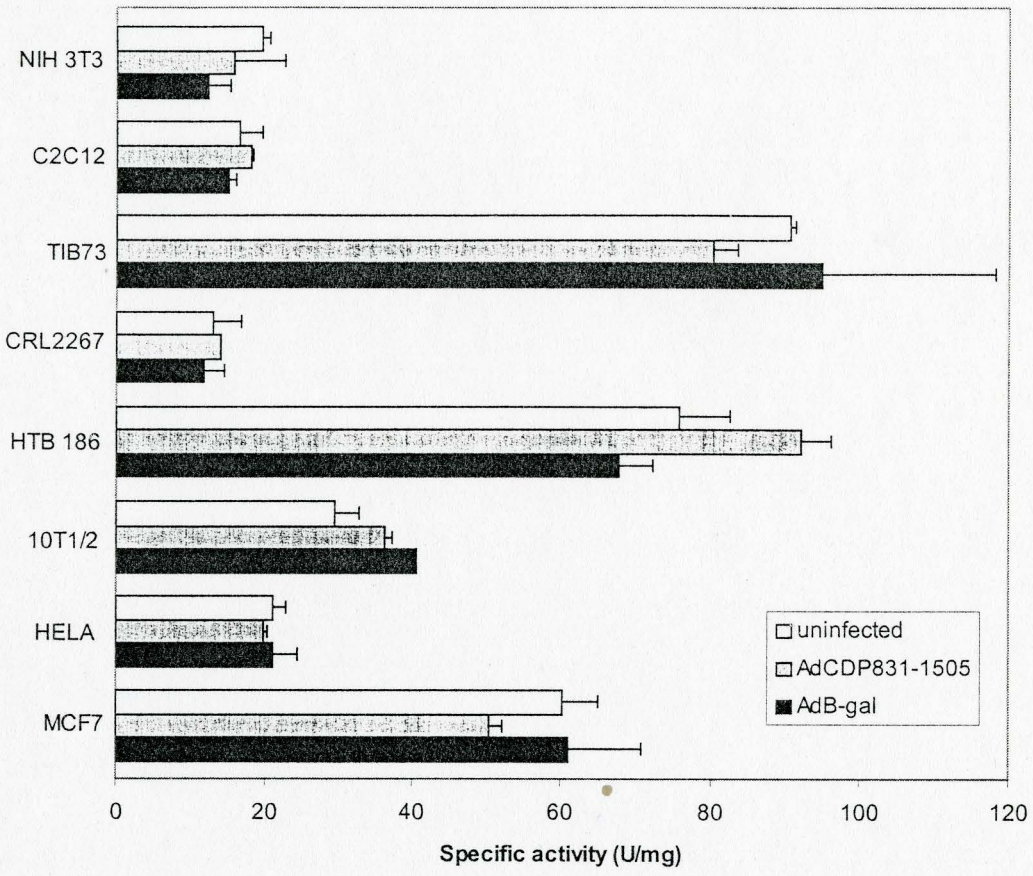


Figure 3.14 Sialidase activity in various cell lines infected with AdCDP$^{831\text{-}1505}$. Three human cell lines (CRL2267, HTB 186, and HELA) and five mouse cell lines were infected with AdCDP831-1505 or Adβ-gal and their lysates assayed for sialidase activity. No significant induction of sialidase activity was observed upon infection with AdCDP831-1505, implying that the CDP831-1505-induced increase in sialidase activity may be restricted to primary human neuronal cells. U = μmol umbelliferone/min.

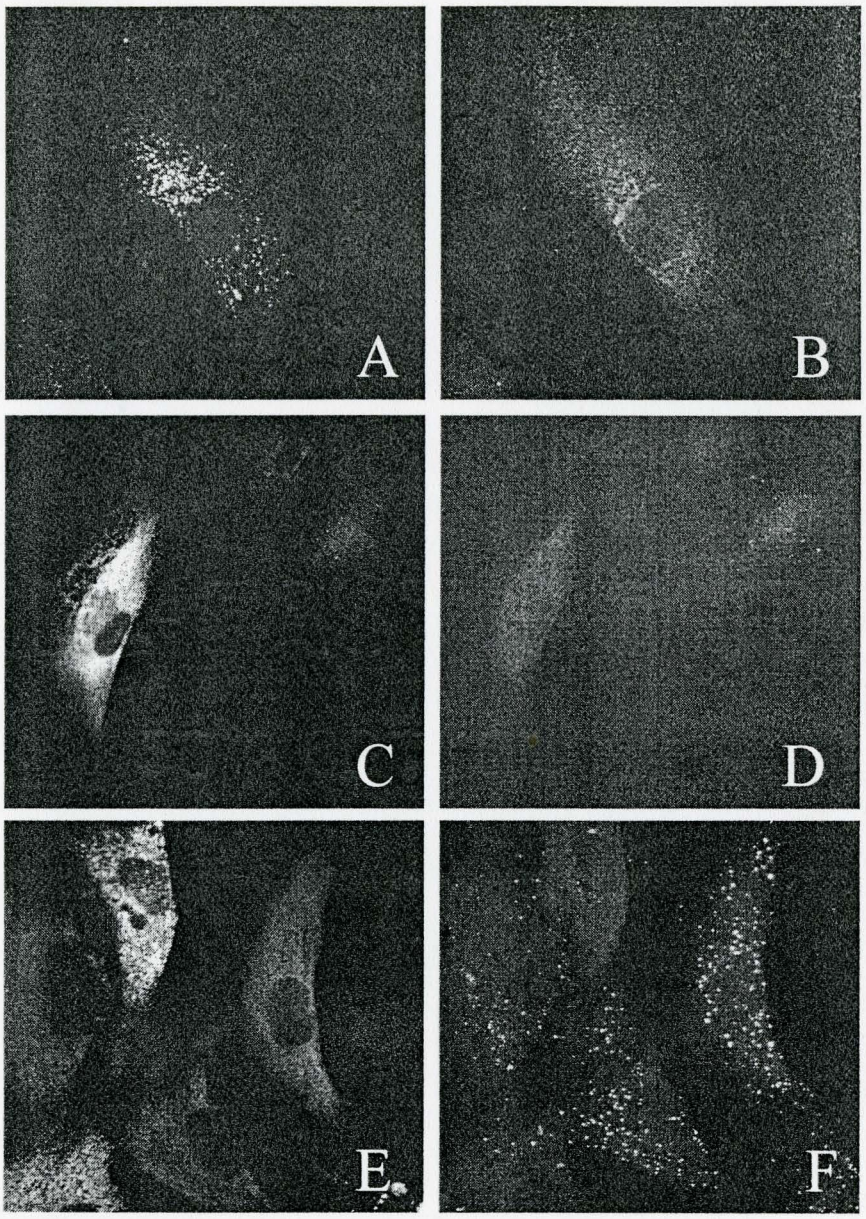
3.10 Reduction of ganglioside accumulation in CRB/TSD cells upon infection with AdCDP$^{831-1505}$.

Since CDP$^{831-1505}$ was observed to up-regulate human lysosomal sialidase promoter activity in CRB cells, resulting in an increase in sialidase expression and activity, the next step was to determine the effect of the increase in sialidase activity on ganglioside GM2 accumulation in Tay-Sachs cells. In order to assess the effect of CDP$^{831-1505}$ on GM2 accumulation in Tay-Sachs cells, CRB/TSD cells plated on coverslips in a 24-well plate were infected with 10^{-2} dilutions of high titre preps of AdCDP$^{831-1505}$, AdCDP$^{1-1109}$, AdSialidase, or AdSialidase^{mut}. AdSialidase^{mut} expresses a mutant form of human lysosomal sialidase that is properly targeted to the lysosome but has been found to have no activity towards Mu-Nana in sialidase assays, presumably due to a point mutation in the highly conserved catalytic region. Seventy-two hrs after infection, the cells were fixed and labelled for analysis by immunocytochemistry. Cells infected with AdSialidase and AdSialidase^{mut}, as well as uninfected cells, were double-labelled with anti-human sialidase and anti-GM2 antibodies, while cells infected with AdCDP$^{831-1505}$ were triple-labelled with anti-human sialidase, anti-GM2, and anti-his-tag antibodies. Cells infected with AdCDP$^{1-1109}$ were also triple labelled with anti-human sialidase, anti-GM2, and anti-HA-tag antibodies. The cells were subsequently incubated with secondary antibodies conjugated to Texas red (anti-sialidase), alexa 488 (green, anti-GM2), or alexa 350 (blue, anti-his-tag on CDP$^{831-1505}$ and anti-HA-tag on CDP$^{1-1109}$). Because the ability of human lysosomal sialidase to reduce GM2 accumulation in Tay-Sachs cells has been confirmed (Igdoura et al., 1999), it was hypothesized that infection with AdCDP$^{831-1505}$ would result in a reduction of GM2 labelling.

Analysis of the uninfected CRB/TSD cells revealed highly variable GM2 labelling, ranging from cells with no labelling at all to those with a great deal of intense and punctate labelling throughout the cytoplasm (Fig. 3.15A). The punctate nature of the labelling confirmed the specificity of the anti-GM2 antibody for GM2-filled lysosomes. Lysosomal sialidase labelling in the cytoplasm of uninfected cells was diffuse rather than punctate, suggesting a certain degree of non-specificity in anti-sialidase antibody binding (the non-specificity of the anti-sialidase antibody was also observed in western blots in the form of numerous extra bands) (Fig. 3.15B) Lysosomal sialidase labelling in AdSialidase infected cells, on the other hand, combined the diffuse cytoplasmic labelling with intense and often "flowery" perinuclear labelling, indicating large amounts of lysosomal sialidase being produced in the endoplasmic reticulum (ER) (Fig. 3.15C). As expected, GM2 labelling in AdSialidase infected cells was less frequent and, when observed, less intense than that of uninfected cells (Fig. 3.15D). Lysosomal sialidase labelling in cells infected with AdSialidase^{mut} was similar to that observed in cells infected with AdSialidase, with diffuse cytoplasmic labelling and more intense perinuclear labelling (Fig. 3.15E). GM2 labelling in AdSialidase^{mut}-infected cells appeared similar to that of uninfected cells (Fig. 3.15F). When analyzed for anti-HA-tag labelling, cells infected with AdCDP$^{1-1109}$ displayed intense, globular labelling immediately surrounding the nuclei (Fig. 3.15G). These results displayed for the first time the inability of CDP$^{1-1109}$ to localize to the nucleus, and these slides were thus excluded from further analysis. Cells infected with AdCDP$^{831-1505}$ displayed brightly labelled nuclei when analyzed for anti-his-tag labelling, indicating the nuclear localization of CDP$^{831-1505}$ (Fig. 3.15H, 3.15J). Lysosomal sialidase labelling in AdCDP$^{831-1505}$-infected cells was

diffuse throughout the cytoplasm with slightly higher intensity around the nucleus, indicating elevated levels of lysosomal sialidase in the ER (Fig. 3.14I). As in AdSialidase-infected cells, GM2 labelling in AdCDP$^{831\text{-}1505}$-infected cells was much less frequent and much less intense than that of uninfected and AdSialidase^{mut}-infected cells (Fig. 3.15K). Though based only on a general examination of the slides, these observations nonetheless provided evidence for the ability of the CDP$^{831\text{-}1505}$-induced increase in lysosomal sialidase levels to reduce ganglioside GM2 accumulation in Tay-Sachs cells.

Figure 3.15 Immunocytochemical analysis of CRB/TSD cells infected with AdSialidase, AdSialidase^{mut}, AdCDP$^{1-1109}$, or AdCDP$^{831-1505}$. CRB/TSD cells were plated on coverslips in a 24-well plate and were infected with 10^{-2} dilutions of high titre preps of AdSialidase, AdSialidase^{mut}, or AdCDP$^{831-1505}$. After 72 hrs the cells were fixed and labelled for analysis. (**A**) Uninfected CRB/TSD cells showed intense, punctate GM2 labelling, indicating a high level of GM2 accumulation in the lysosomes. (**B**) Sialidase labelling in uninfected cells was non-punctate and diffuse throughout the cytoplasm. (**C**) Infection with AdSialidase resulted in the production of large amounts of lysosomal sialidase in the ER, as evidenced by intense and profuse anti-sialidase labelling around the nuclei of infected cells. (**D**) GM2 stores were reduced in AdSialidase infected cells, evidenced here by the lack of GM2 labelling in the infected cell and the persistent GM2 labelling in the uninfected cell (above right). Infection with AdSialidase^{mut} also resulted in the production of a large amount of transgenic protein (**E**), but GM2 stores remained high, presumably due to the mutant enzyme's lack of catalytic activity (**F**). Cells infected with AdCDP$^{1-1109}$ showed intense, globular anti-HA-tag labelling in the cytoplasm, indicating the inability of CDP$^{1-1109}$ to localize to the nucleus (**G**). Infection with AdCDP$^{831-1505}$ resulted in intense nuclear labelling with an anti-his-tag antibody (**H**, **J**), confirming the nuclear localization of transgenic CDP$^{831-1505}$. (**I**) AdCDP$^{831-1505}$-infected cells also displayed slightly more intense lysosomal sialidase labelling immediately surrounding the nucleus, suggesting an elevated level of sialidase production in the ER. GM2 labelling was reduced in AdCDP$^{831-1505}$-infected cells (**K**), indicating that the elevated levels of lysosomal sialidase caused by CDP$^{831-1505}$ were sufficient to deplete GM2 accumulation in Tay-Sachs cells.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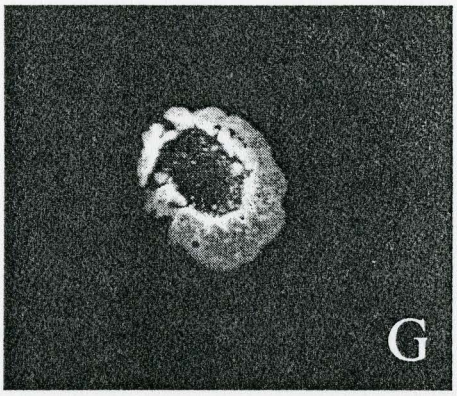
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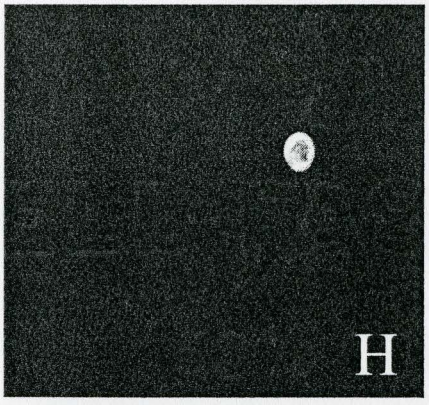
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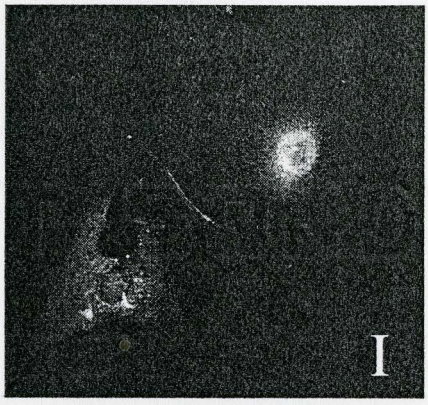
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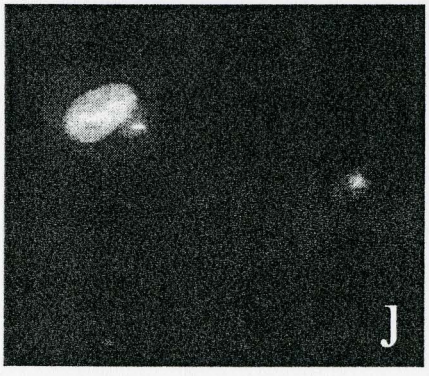
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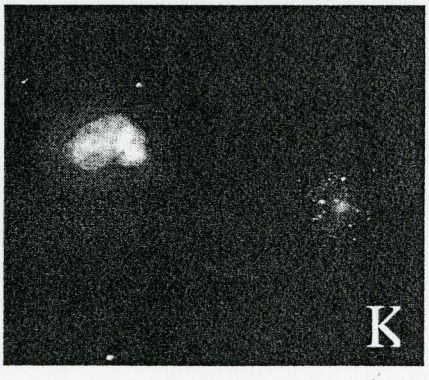
K

In order to determine if the level of GM2 accumulation was, in fact, reduced by infection with AdCDP$^{831\text{-}1505}$, cells were each assigned a rank based on their intensity of GM2 labelling (*** = high intensity; ** = moderate intensity; * = low intensity; 0 = no labelling). The effect of each treatment on GM2 accumulation could therefore be quantified by observing differences in the proportions of cells of each rank. Results of this analysis corroborated the general observations discussed previously (Fig. 3.15). As expected, the uninfected cell population contained a high proportion of labelled cells, with greater than 50% of the cells displaying punctate labelling of ganglioside GM2-filled lysosomes. Most significantly, greater than 20% of the uninfected cells displayed high to moderate intensity GM2 labelling, indicating a high amount of GM2 accumulation. Cells infected with AdSialidase showed a marked decrease in high and moderate intensity GM2-labelled cells with a corresponding increase in unlabelled cells, indicating the successful catabolism of ganglioside GM2 by transgenic lysosomal sialidase. Cells infected with AdSialidase^{mut} had a proportion of high and moderate intensity GM2-labelled cells similar to that of the uninfected cells (~20%), but the proportion of low intensity labelled cells was approximately 10% lower than that of the uninfected cells, an unexpected result. Despite its confirmed inactivity towards synthetic substrates in enzyme activity assays, it is possible that the mutant sialidase expressed by AdSialidase^{mut} retains some residual activity towards ganglioside GM2 and, when present in large amounts, may have the ability to reduce GM2 accumulation in the lysosome, albeit at severely reduced efficiency. Interestingly, the cells infected with AdCDP$^{831\text{-}1505}$ showed the greatest reduction in proportion of GM2-labelled cells; the proportion of labelled cells in this group was approximately 25% lower than that of

uninfected cells. Accompanying this reduction in GM2-labelled cells was an increase in the proportion of unlabelled cells to > 70%. The reduction of GM2-labelled cells in the AdCDP$^{831\text{-}1505}$-infected population was greater than that of the AdSialidase-infected group, suggesting that the elevated levels of lysosomal sialidase that arise from stimulation of the endogenous gene are more effective at clearing GM2 stores than those brought about by sialidase transgene expression. Over all, the results of this immunocytochemical analysis provide evidence for the ability of CDP$^{831\text{-}1505}$ to elevate endogenous lysosomal sialidase in CRB/TSD cells to a level sufficient to facilitate the clearance of ganglioside GM2 from diseased lysosomes.

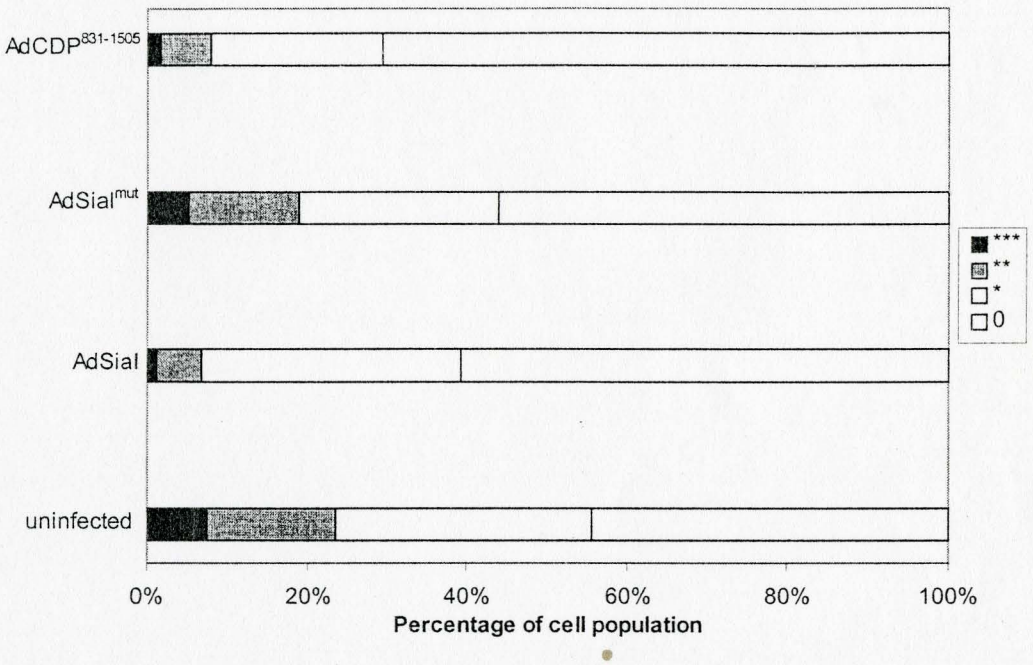

Figure 3.16 GM2 labelling in CRB/TSD cells infected with AdSialidase, AdSialidase^{mut}, or AdCDP$^{831-1505}$. Labelling with the anti-ganglioside GM2 antibody in infected and uninfected CRB/TSD cells was ranked according to intensity (*** = high; ** = moderate; * = low; 0 = no labelling). The number of cells of each rank are displayed as proportions of the total cell population. Infection with AdCDP$^{831-1505}$ led to a significant decrease in the proportion of labelled cells, indicating the ability of CDP$^{831-1505}$ to induce lysosomal sialidase expression to a level sufficient to reduce GM2 accumulation in diseased cells.

Though the effect of AdCDP$^{831\text{-}1505}$ infection on GM2 stores in Tay-Sachs cells had been established, it was nonetheless necessary to examine more closely the relationship between the levels of CDP$^{831\text{-}1505}$ and levels of lysosomal sialidase and ganglioside GM2 in infected cells. To accomplish this, 46 AdCDP$^{831\text{-}1505}$-infected cells were individually scored for their intensity of CDP labelling, lysosomal sialidase labelling, and GM2 labelling (3 = high intensity; 2 = moderate intensity; 1 = low intensity; 0 = no labelling) - presumably, the intensity of the immunolabelling of a target molecule is directly related to its amount in the cell. With the amounts of the three target molecules quantified in the AdCDP$^{831\text{-}1505}$-infected cells, relationships between the molecules could subsequently be quantified.

The first relationship examined was the one between CDP$^{831\text{-}1505}$ and lysosomal sialidase. Previous experiments have indicated that infection with AdCDP$^{831\text{-}1505}$ results in an increase in lysosomal sialidase expression - our hypothesis for this analysis, therefore, was that the level of transgenic CDP$^{831\text{-}1505}$ in the cell would correspond with the level of lysosomal sialidase, i.e., in a given cell, the CDP score would be similar or equal to the lysosomal sialidase score. The results of this analysis supported our hypothesis – of the 46 cells scored, 28 (~ 61%) had CDP scores equal to their sialidase scores and a further 15 (~ 33%) had a difference of only one between the two scores (Fig. 3.17). These results suggest that lysosomal sialidase levels may be increased by CDP$^{831\text{-}1505}$ in a dose-dependent manner.

The second relationship analyzed was the one between CDP$^{831\text{-}1505}$ and ganglioside GM2 levels – we hypothesized that this relationship was also one of dose-dependence, but that the amount of GM2 accumulation would be inversely related

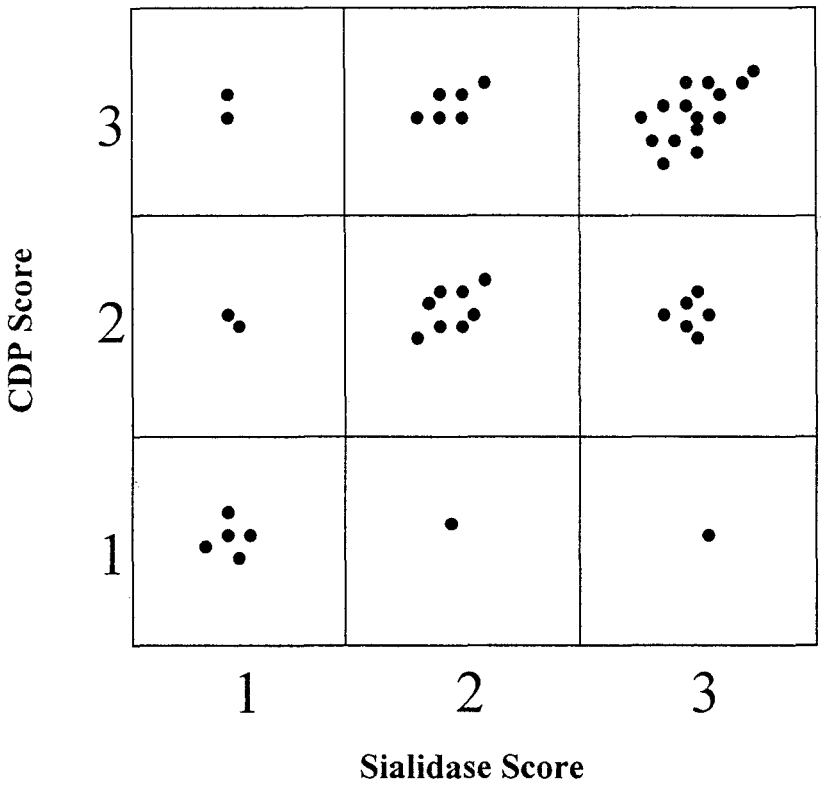

Figure 3.17 Analysis of the relationship between CDP$^{831\text{-}1505}$ and the level of lysosomal sialidase in AdCDP$^{831\text{-}1505}$-infected CRB/TSD cells. 46 AdCDP$^{831\text{-}1505}$-infected cells were scored for their intensity of CDP labelling and lysosomal sialidase labelling (3 = high intensity; 2 = moderate intensity; 1 = low intensity; 0 = no labelling). In the majority (~ 94%) of the cells, there was either no difference or a difference of only one between the CDP score and the sialidase score, indicating that lysosomal sialidase levels increase in response to CDP$^{831\text{-}1505}$ in a dose-dependent manner. Each point in the above grid represents one scored cell (n = 46).

to the level of $CDP^{831-1505}$ present in the cell - high CDP scores would correspond with low GM2 scores, and the differences between the scores would therefore be high. Though the relationship did not appear entirely linear, results of the scoring analysis were in agreement with an inverse dose-dependent relationship between $CDP^{831-1505}$ and GM2 levels, as the majority (92%) of cells with CDP scores of 3 or 2 had a GM2 score of zero (Fig. 3.18).

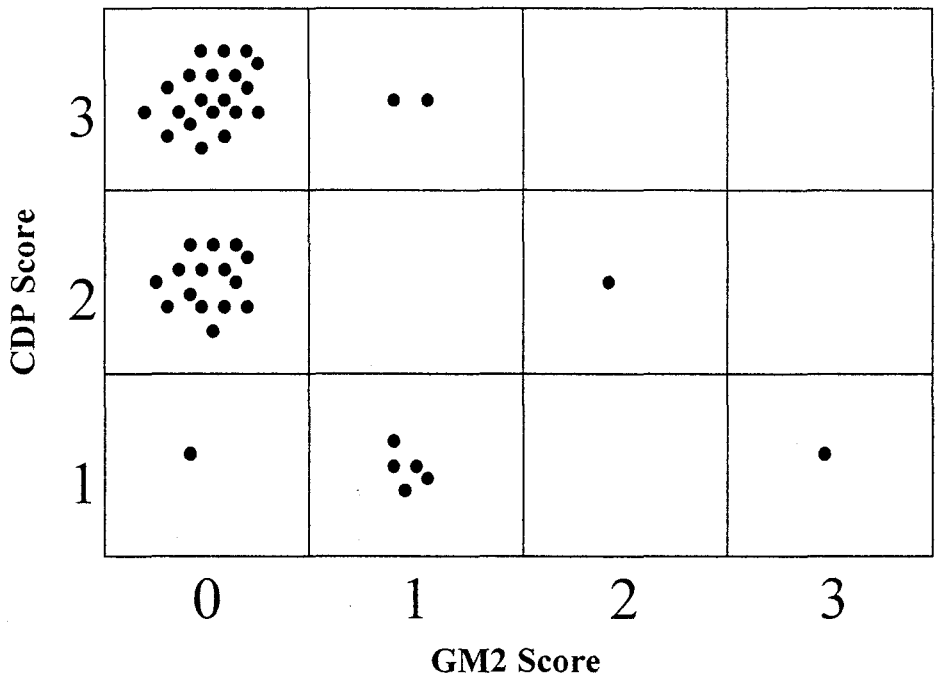

Figure 3.18 Analysis of the relationship between CDP$^{831-1505}$ and ganglioside GM2 levels in AdCDP$^{831-1505}$-infected CRB/TSD cells. 46 AdCDP$^{831-1505}$-infected cells were scored for their intensity of CDP labelling and ganglioside GM2 labelling (3 = high intensity; 2 = moderate intensity; 1 = low intensity; 0 = no labelling). The majority (92%) of cells with CDP scores of 3 or 2 had a GM2 score of zero, indicating an inverse dose-dependent relationship between CDP$^{831-1505}$ and GM2 levels. Each point in the above grid represents one scored cell (n = 46).

3.11 Analysis of the ganglioside composition of AdCDP$^{831-1505}$-infected CRB/TSD cells by ganglioside extraction and thin layer chromatography (TLC).

In order to complement the immunocytochemical analysis and confirm the reduction of ganglioside GM2 accumulation in CRB/TSD cells brought about by infection with AdCDP$^{831-1505}$, an analysis of the ganglioside composition of CRB/TSD cells infected with AdCDP$^{831-1505}$ was required. CRB/TSD cells in 100 mm dishes were first incubated with media containing L-[3-^{3}H] serine – because the first step in ganglioside biosynthesis involves the condensation of L-serine and palmitoyl-CoA, reactions involving L-[3-^{3}H] serine would result in the production of gangliosides labelled with ^{3}H. After three days of incubation with radioactive media, the cells were infected with 10^{-2} dilutions of high titre preps of AdCDP$^{831-1505}$, AdCDP$^{1-1109}$, or AdEGFP. The cells were maintained in radioactive media for three days post-infection, after which time the cells were harvested and subjected to the ganglioside extraction protocol. Ganglioside extracts were subsequently run on a TLC plate and exposed to autoradiographic film. It was expected that the signal corresponding to GM2 in the AdCDP$^{831-1505}$-infected lane would be reduced compared to the GM2 signals in the other lanes, as the other viruses were expected to have no effect on GM2 stores. Unfortunately, the developed film contained no signal in any of the lanes (not shown). When exposure to autoradiographic film yielded no visible results, gangliosides on the TLC plate were stained by spraying the plate with a resorcinol/HCl solution and heating it at 110°C for ~ 24 hrs. No staining was observed in any of the extract lanes, though staining of a purified GM2 standard was slightly visible (Fig. 4.15). These results suggest that the extraction protocol did not yield enough gangliosides to be visualized by staining or autoradiography.

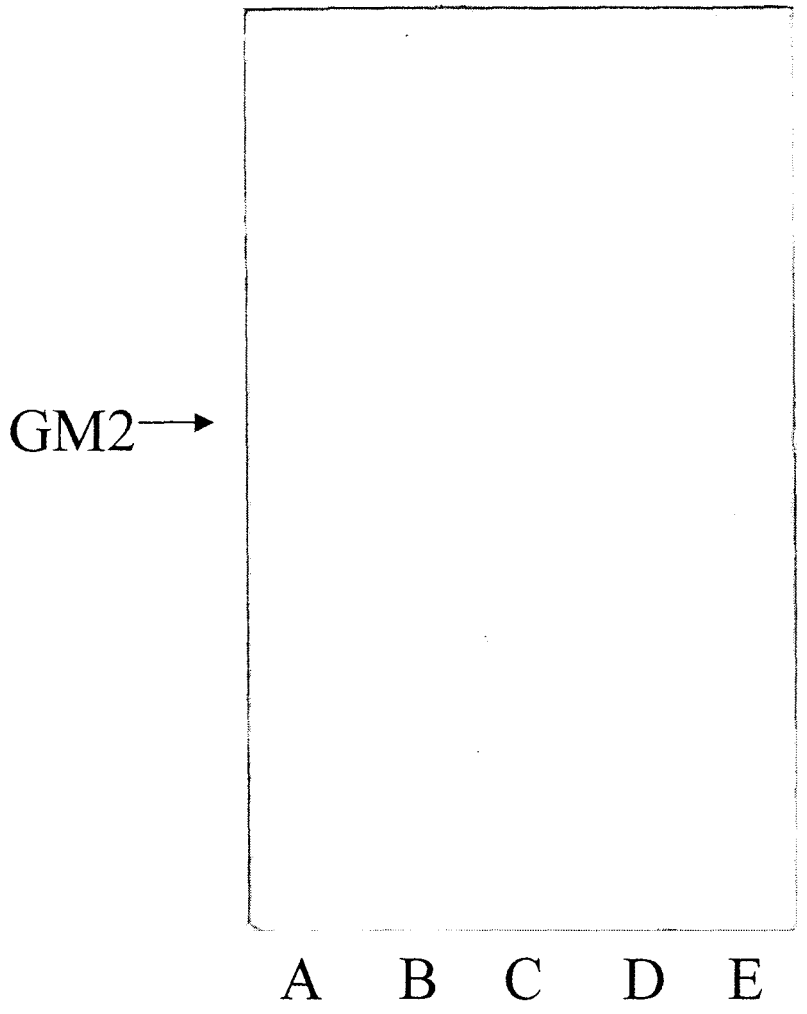

Figure 3.19 TLC of ganglioside extracts from cells infected with AdCDP$^{831\text{-}1505}$, AdCDP$^{1\text{-}1109}$, or AdEGFP. Gangliosides were extracted from infected CRB/TSD cells and separated by TLC. After staining with resorcinol/HCl, only a slight staining of a standard of purified GM2 was visible on the plate (lane A). No bands were visible in the lanes corresponding to the extracts of uninfected cells (lane B), AdCDP$^{831\text{-}1505}$-infected cells (lane C), AdCDP$^{1\text{-}1109}$-infected cells (lane D), or AdEGFP-infected cells (lane E). These results suggest that the ganglioside extraction from CRB/TSD cells was unsuccessful.

CHAPTER 4

Discussion

The GM2 gangliosidoses constitute a group of catastrophic neurodegenerative disorders characterized by the intralysosomal accumulation of ganglioside GM2. Depending on the type and severity of the genetic defect responsible, the disease can manifest in the form of various clinical symptoms, including mental and physical retardation, blindness, seizures, and psychological disorders. In the most severe forms of GM2 gangliosidosis, namely Tay-Sachs disease and Sandhoff disease, affected individuals rarely survive beyond 4 years of age. Tay-Sachs disease is caused by mutations in the α-subunit of β-hexosaminidase A (Hex A), a lysosomal acid hydrolase responsible for the degradation of ganglioside GM2. This deficiency of Hex A results in the intralysosomal accumulation of ganglioside GM2 in cells of the central nervous system, ultimately leading to the premature death of the cells and pervasive neurodegeneration. Although widespread screening for Tay-Sachs mutations in susceptible populations has greatly decreased the incidence of the disease, Tay-Sachs disease remains a terminal disorder for which no effective treatment or cure exists.

The development of a mouse model of Tay-Sachs disease was an important step in the quest for a cure. The *Hexa-/-* mouse was unusual, however, in that it displayed none of the symptoms associated with the human disease. In fact, the *Hexa-/-* mice showed no apparent difference in size, behaviour, or reproductive ability compared to their wild-type littermates for up to 1 year. This unexpected phenotype led to the discovery that *Hexa-/-* mice are able to bypass their Hex A deficiency by the action of lysosomal sialidase. It was found that, in addition to its known substrate, ganglioside GM3, mouse lysosomal sialidase is also able to catalyze the degradation of ganglioside GM2 to GA2, which in turn proceeds normally through the remainder

of the ganglioside catabolic pathway. This sialidase-mediated bypass of Hex A deficiency is not functional in humans for unknown reasons. Upon discovery of the bypass in *Hexa-/-*, researchers speculated that the bypass was due either to a higher affinity of mouse lysosomal sialidase for GM2 or to higher enzyme levels. Recent evidence suggests that the difference between human and mouse lysosomal sialidases lies in the level of expression rather than substrate specificity, for overexpression of human lysosomal sialidase has been observed to effectively reduce GM2 accumulations in human Tay-Sachs neuroglia cells (Igdoura et al., 1999). This study also suggested for the first time that pharmacological stimulation of endogenous lysosomal sialidase might have therapeutic benefit in Tay-Sachs disease. Unfortunately, the regulatory mechanisms controlling human lysosomal sialidase expression have not yet been fully elucidated. The primary purpose of this research was to begin the study of human lysosomal sialidase regulation, with the hopes that a mechanism for induction of sialidase expression might arise. The ultimate goal remains to identify potential targets for the pharmacological induction of endogenous lysosomal sialidase and the development of an effective treatment for Tay-Sachs disease and related Hex A deficiency disorders.

The comparison of human and mouse lysosomal sialidase promoter-reporter constructs in both human and mouse cell lines provides further evidence for an inherent difference in expression levels in the two species. The approximately 6-7-fold higher activity of the mouse promoter-reporter construct in both cell lines indicates that the mouse lysosomal sialidase promoter is capable of driving transcription at a much higher rate than the human promoter. This suggests that the higher levels of lysosomal sialidase that give rise to the sialidase-mediated bypass of

Hex A deficiency in mice may be at least partly due to a more active promoter. This difference in basal promoter activity is not unexpected, for although the promoters share numerous regions of high homology, a significant amount of divergence appears to have occurred in the lysosomal sialidase regulatory sequence, particularly in the region directly adjacent to the transcriptional start (Fig. 1.3). While these results implicate the difference in basal lysosomal sialidase promoter activity as the reason why the sialidase-mediated bypass of Hex A deficiency is functional in mice but not in humans, they do not rule out the possibility that post-translational events may also contribute, or that a difference in the two enzymes' affinities for GM2 might exist.

The relative activities of the human lysosomal sialidase promoter deletion constructs provide significant insight into the structure of the sialidase regulatory region, as they clearly indicate regions containing the enhancer and silencer elements important to lysosomal sialidase transcription. One of the silencer elements believed to play a major role in the regulation of human lysosomal sialidase expression was the CDP binding site present ~ 1 kb upstream of the transcription initiation site. However, removal of this site does not appear to affect promoter activity, as del2kb and del2kb-CDP display no appreciable difference in activity *in vitro*. Though this CDP binding site has been excluded as being important to human lysosomal sialidase promoter activity, the ability of CDP to bind to other sites in the promoter and affect sialidase expression has not been ruled out.

In all three cell lines, a substantial difference in activity is observed between the 2.9 kb promoter-reporter construct (fullprom) and the 2 kb promoter-reporter construct (del2kb), with the activity of del2kb approximately 2-5-fold greater than that of fullprom. These results suggest the existence of one or more important silencer

elements in the region 2-2.9 kb upstream of the transcription initiation site. Interestingly, analysis of the human promoter sequence with an updated version of MatInspector revealed the presence of two additional putative binding sites for CDP in the 2-2.9 kb region. In addition, while the region in which the sites reside in the human promoter is highly homologous with the corresponding region in the mouse promoter, one of the sites is absent from the mouse sequence. It is therefore possible that the lack of this particular CDP binding site in the mouse promoter is at least partially responsible for its higher activity, while its presence in the human promoter results in its lower activity. In order to fully assess the importance of this CDP binding site to human lysosomal sialidase expression, further studies of the human promoter should include a comparison of full-length promoter activity with that of a promoter deletion construct from which the site is removed.

It is apparent that a number of important elements exist within 2 kb of the transcription initiation site. The deletion construct containing ~ 1.5 kb of promoter sequence (del1.5kb) is the most active construct in all three cell types. Activity then drops dramatically upon deletion of the promoter sequence to 1 kb, only to increase again upon further deletion to 700 bp. These results suggest the presence of one or more enhancer elements between 1-1.5 kb, a powerful silencer between 700 bp-1 kb, and one or more enhancers within 700 bp of the transcription initiation site. Analysis of these regions reveals the presence of numerous regulatory elements, including binding sites for MyoD and the NeuroD factors. Though the effect of these factors on promoter activity was found to be minimal, are more stringent analysis of the regulatory elements in these key regions is needed to determine their importance to lysosomal sialidase expression.

Though it is not clear where in the human lysosomal sialdiase promoter they bind or by what mechanism they operate, truncations of the transcription factor CDP have a clear and consistent effect on promoter activity *in vitro*. In contrast with CDP's accepted reputation as a transcriptional repressor, co-transfection of the promoter-reporter construct with full-length CDP leads to a slight increase in promoter activity. Two explanations exist for this unexpected result: (1) CDP acts as a transcriptional activator in the context of the human lysosomal sialidase promoter, or (2) the transgenic CDP is interacting with other transcription factors, thereby altering the regulatory environment in such a way as to lead to elevated promoter activity. Both explanations are equally plausible, for CDP is known for its ability to act as both a transcriptional repressor and activator, and CDP has also been observed to bind to and sequester the neuronal transcriptional repressor SATB1, thereby reducing the repressor activity of both factors (Liu et al., 1999). A binding site for SATB1 is present in the human promoter, lending credence to the second hypothesis, though a more thorough understanding of CDP and its interaction with DNA and other transcription factors is required to fully resolve the mechanism of its stimulation of the lysosomal sialidase promoter.

Despite the results of the promoter/CDP truncation co-transfection experiments, CDP could not be considered to have a major role in the regulation of endogenous lysosomal sialidase expression in neuronal tissue unless its expression in neuronal tissue could be confirmed. The results of the RT-PCR experiments in CRB/Nor and CRB/TSD total RNA succeeded in doing this – both cell lines were found to express CDP. These experiments also ruled out a role for Cux-2, a supposed neuronal-specific CDP homolog, in lysosomal sialidase expression in CRB/Nor and

CRB/TSD cells. Due to the embryonic origin of the CRB cell lines, these results cannot confirm the persistent expression of CDP upon terminal differentiation of cells. They do, however, suggest a role for CDP in the regulation of lysosomal sialidase expression during development.

Of all the CDP truncations tested, the most profound change in promoter activity occurs upon co-transfection with $CDP^{831-1505}$. This particular truncation leads to a near 50-fold increase in activity compared to that of the promoter alone. The consistency of the up-regulation observed upon co-transfection with $CDP^{831-1505}$ indicated a genuine ability of this construct to increase human lysosomal sialidase promoter activity by a significant amount. The mechanism of this up-regulation, however, remains to be elucidated. Because this construct is missing CR1, and is therefore considered unable to carry out its repressive action by CCAAT box displacement, it is possible that $CDP^{831-1505}$ acts as a "dominant negative" – the truncated CDP is able to out-compete endogenous full-length CDP for binding to the CDP binding site, but its inability to repress promoter activity by CCAAT box displacement results in an increase in promoter activity rather than a decrease. The other possibility is that the truncation acts as a direct activator of the promoter – perhaps the combination of DNA-binding domains in $CDP^{831-1505}$ result in a unique ability to bind to promoter elements, thereby allowing it to activate gene expression. In support of this hypothesis is the observation that a naturally occurring 110 kDa isoform of CDP, which is comprised of the same combination of DNA-binding domains as $CDP^{831-1505}$ (Figs. 1.4 and 3.7), acts as a transcriptional activator *in vitro* (Moon et al., 2001).

Regardless of the mechanism, it is apparent that the promoter up-regulation caused by $CDP^{831-1505}$ results in a corresponding increase in lysosomal sialidase activity. Though results of Western blot analyses could not directly confirm an elevated level of protein (it is possible that the ambiguous results observed with this Western blot analysis were caused by an incomplete transfer of protein from the gel to the nitrocellulose membrane, as the bands in central region of the gel are faint and irregular), we nonetheless observed a significant increase in sialidase activity in CRB/Nor and CRB/TSD cells upon infection with $AdCDP^{831-1505}$. In a way, this increase in enzyme activity is indicative of an elevated level of lysosomal sialidase, for the rate of an enzyme-catalyzed reaction can only be raised by increasing the amount of enzyme present in the reaction (enzyme activity can also be elevated by increasing the enzyme's affinity for its substrate, but $CDP^{831-1505}$ is unlikely to have caused this). A more sensitive method for the detection of increased gene expression, such as quantitative real-time PCR or Northern blot, is likely required to directly confirm the effect of $CDP^{831-1505}$ on lysosomal sialidase expression.

The results of the sialidase assays of various transformed human and mouse cell lines infected with $AdCDP^{831-1505}$ seems to indicate that the $CDP^{831-1505}$-induced increase in sialidase activity may be restricted to primary cells. This was not an unexpected result, as the regulatory environments in most transformed cell lines are considered to be quite abnormal. In order to assess the true tissue and/or cell-type specificity of the $CDP^{831-1505}$-induced increase in sialidase activity, future experiments should include numerous primary human cell lines with varying tissue origins.

The effect of $CDP^{831-1505}$ on lysosomal sialidase expression raises a very important question – is the up-regulation specific to lysosomal sialidase, or does

$CDP^{831-1505}$ up-regulate the expression of other genes as well? In order to pursue $CDP^{831-1505}$ as a potential therapeutic agent in the treatment of Hex A-deficient GM2 gangliosidoses, this question should most definitely be answered. Future extensions of this research will therefore likely include comparative DNA microarray analysis of uninfected and $AdCDP^{831-1505}$-infected cells.

The dramatic increase in lysosomal sialidase activity observed in CRB/TSD cells upon infection with $AdCDP^{831-1505}$ was a very encouraging result – if it is possible to increase lysosomal sialidase activity in human Tay-Sachs cells, then it might also be possible to activate the sialidase-mediated bypass and reduce GM2 accumulation. Results of the immunocytochemical analysis of $AdCDP^{831-1505}$-infected CRB/TSD cells seem to indicate that the reduction of GM2 accumulation is indeed a beneficial consequence of treatment with $CDP^{831-1505}$. General observation of $AdCDP^{831-1505}$-infected cells revealed an increase in lysosomal sialidase labelling and a reduction in frequency and intensity of ganglioside GM2 labelling, indicating both an increase in lysosomal sialidase expression and a decrease in GM2 accumulation. In fact, upon quantitation of the intensity of GM2 labelling, it was found that $CDP^{831-1505}$ reduced GM2 accumulation to a greater degree than transgenic sialidase – the $AdCDP^{831-1505}$-infected population contained approximately 10% fewer GM2-labelled cells than the AdSialidase-infected population. This is likely due to the fact that the elevated lysosomal sialidase levels in $AdCDP^{831-1505}$-infected cells are the result of stimulation of the endogenous gene promoter, and are thus subject to normal cellular regulation. In this case, lysosomal sialidase levels remain within physiological limits and do not interfere with other cellular processes. Conversely, infection with AdSialidase results in the profuse and unregulated expression of lysosomal sialidase,

which has been observed to have cytotoxic consequences; it is believed that without the concurrent addition of transgenic protective protein/cathepsin A (PPCA), large amounts of transgenic sialidase cannot be properly targeted to the lysosome and thus remain in the ER. As a result, not only is the transgenic sialidase unable to travel to the lysosome to perform its metabolic function, but its retention in the ER is thought to cause a disruption of normal cellular function. Aside from verifying the ability of $CDP^{831\text{-}1505}$ to activate a sialidase-mediated bypass capable of reducing GM2 stores in Tay-Sachs cells, these results suggest that stimulation of the endogenous lysosomal sialidase gene as a treatment for Hex A-deficient GM2 gangliosidoses may prove to be a more effective strategy than typical gene replacement therapy.

Further examination of the relationships between $CDP^{831\text{-}1505}$, lysosomal sialidase levels, and GM2 accumulation seems to reveal a certain degree of dose-dependence – in the immunocytochemical analysis of infected CRB/TSD cells, high levels of $CDP^{831\text{-}1505}$ were associated with relatively high levels of lysosomal sialidase and greatly reduced GM2 stores. This information will become important as more factors with the potential to up-regulate sialidase are discovered and tested for their efficiency in clearing GM2 stores in Tay-Sachs cells. In the end, the factor capable of eliciting an effective clearance of GM2 at the lowest dose will likely be pursued as a therapeutic up-regulation mechanism.

It is unknown just how much ganglioside GM2 clearance from Tay-Sachs lysosomes is necessary to restore normal function and prevent cell death. This information will become critical in the coming years as potential therapies for Hex A-deficient GM2 gangliosidoses are proposed and developed. It can be assumed, however, that if GM2 stores can be brought down to levels such that the ganglioside

profile of a Tay-Sachs cell resembles that of a normal cell, then normal cellular function will likely return. With these notions in mind, and to complement the results of our immunocytochemistry experiments, we attempted to resolve the ganglioside profile of Tay-Sachs cells infected with $AdCDP^{831-1505}$ by extracting the total ganglioside content from the cells and separating it by TLC. Unfortunately, the ganglioside extraction protocol used was unsuccessful for these purposes, and will therefore likely require modification in future attempts. Of course, even if ganglioside GM2 accumulation in Tay-Sachs cells can be brought down to relatively normal levels by a potential therapeutic agent, assessment of the treatment in *in vivo* models will be necessary in order to determine its effect on cellular function and overall phenotype.

As is the case with all diseases, the ultimate goal of the study of the GM2 gangliosidoses is to discover and develop an effective treatment or, ideally, a cure. The discovery of the lysosomal sialidase-mediated bypass of Hex A deficiency in Hex A-knockout mice was a momentous step in this long journey, for it showed for the first time that an alternative, naturally occurring biochemical pathway existed for the metabolism of ganglioside GM2, the normally innocuous plasma membrane component that accumulates to cytotoxic levels in the lysosomes of Hex A-deficient neurons. It was believed then, as it is now, that if this bypass could somehow be activated in human Tay-Sachs patients, than it may be possible to reduce the amount of GM2 accumulation and alleviate, or halt altogether, the neurodegeneration that characterizes the disease. One potential method by which the bypass could be generated in humans is by the stimulation of endogenous lysosomal sialidase expression. When applicable, induction of alternate gene product expression to account for the defect in an inherited disorder has become an increasingly appealing

alternative to typical gene therapy; therapies for Duchenne muscular dystrophy, sickle cell disease, and β-thalassaemia are currently being developed using this approach.

If a sialidase-mediated bypass of Hex A deficiency is to be activated in humans, information regarding the regulation of human lysosomal sialidase expression will be of the utmost importance. The work presented here represents a modest beginning to the characterization of the human lysosomal sialidase promoter and the search for strategies to stimulate the promoter and up-regulate the sialidase gene. Just as the potential of $CDP^{831-1505}$ to stimulate lysosomal sialidase expression and reduce GM2 stores was discovered with this research, we are hopeful that, as this work is continued, more regulatory elements will arise as potential targets for the pharmacological stimulation of the lysosomal sialidase gene. Once these molecular targets are revealed, a safe and effective treatment for the Hex A-deficient GM2 gangliosidoses cannot be far removed.

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