HETEROALLELISM, SCREENING AND STRUCTURE-FUNCTION STUDIES AT THE *HEXA* LOCUS

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PREFACE

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ABSTRACT

Tay-Sachs disease (TSD) is a recessively inherited disorder characterized by the pathological accumulation of G_{M2} ganglioside in neuronal cells. Mutations in the *HEXA* gene, which codes for the α-subunit of hexosaminidase A (Hex A), cause TSD. The objectives of this thesis are all related to the genetics of TSD and to the properties of the *HEXA* gene product.

Only three *HEXA* mutations account for >90% of mutant TSD alleles in the Ashkenazi Jewish population, a high risk group. The use of DNA diagnostics was therefore evaluated as an alternative to the conventional TSD screening (enzyme) test. Our study indicated that neither test has a sensitivity of 1 and that both methods used iteratively improve the predictive value of either test alone.

To determine the molecular basis of the observed phenotypic heterogeneity in TSD, four novel *HEXA* mutations were identified by SSCP (single stranded conformation polymorphism) analysis; R170W, a CT deletion at positions 927-928 or 929-930, D258H and IVS7, -7 G->A. D258H was the first putative active site mutation identified in exon 7. The impact of the IVS7, -7 G->A mutation on the formation of accurately spliced *HEXA* mRNA was investigated. Competitive PCR was used to determine the effect of this mutation and a known mutation (G269S) on gene expression.

A novel expression system was established in a TSD neuroglial cell line (TSD-NG) for the study of TSD mutations. TSD-NG cells were transiently transfected with *HEXA* cDNA's carrying mutations responsible for the acute, sub-acute and chronic forms of TSD. The percent residual Hex A activity in transfected TSD-NG cells is similar to that in patient's fibroblasts.

The TSD-NG system was also used to investigate three candidate active site residues (E307, E323, E462) of the α-subunit of Hex A. These residues were chosen on the basis of their conservation among glycosidases of family #20, one of whose members was recently crystallized. Our study suggests that E323 and E462 are located in the active site of Hex A and that E323 is a catalytic residue.

RÉSUMÉ

La maladie de Tay-Sachs est un désordre autosomique récessif caracterisé par l'accumulation pathologique de G_{M2} ganglioside dans les cellules neuronales. Des mutations dans le gène *HEXA*, codant pour la sous-unité α de l'enzyme hexosaminidase A (Hex A), causent la maladie de Tay-Sachs. Les objectifs de cette thèse sont reliés à la génétique de la maladie de Tay-Sachs ainsi qu'a certaines propriétés biochimiques de l'enzyme Hex A.

Seulement trois mutations *HEXA* comptent pour plus de 90% des allèles mutantes dans la population juive ashkénaze, un groupe à risque élevé. L'utilisation de l'ADN comme outil diagnostique a été évalué comme alternative afin de remplacer la méthode enzymatique conventionnelle. Nos résultats indiquent qu'aucun de ces tests (ADN ou enzymatique) a une sensibilité égale à 1, malgré que lorsqu'ils sont utilisés ensembles, ces tests améliorent la valeur prédictive (comparativement à la valeur obtenue lorsque ces tests sont faits séparément).

Afin de déterminer la base moléculaire de l'hétérogénéité phénotypique observée dans la maladie de Tay-Sachs, quatre mutations dans le gène *HEXA* ont été identifiées par la méthode de SSCP (Single Strand Conformation Polymorphism): R170W, la délétion des nucléotides CT aux positions 927-928 ou 929-930, D258H, et IVS7, -7G->A. La mutation D258H a été la première mutation identifiée dans l'exon 7 du gène *HEXA*

impliquant un site actif hypothétique. L'impact de la mutation IVS7, -7G->A sur l'épissage de l'ARNm *HEXA* a été analysé. La méthode de PCR compétitif a été utilisée afin de déterminer l'effet de cette mutation ainsi qu'une autre (G269S) sur l'expression du gène *HEXA*.

Un nouveau système d'expression a été mis au point dans une souche établie de cellules neurogliales provenant d'un patient Tay-Sachs afin d'étudier les mutations dans le gène *HEXA*. Les cellules neurogliales ont été transfectées de façon transitoire avec un ADNc *HEXA* contenant des mutations responsables pour les formes cliniques suivantes: aigüe, sous-aigüe et chronique. Le pourcentage d'activité résiduelle de l'enzyme Hex A mesuré dans les cellules transfectées est similaire a celui retrouvé dans les fibroblastes des patients Tay-Sachs.

Le système d'expression a finalement été utilisé afin d'analyser trois acides aminés candidats pour être impliqués dans le site actif de la sous unité α de l'enzyme Hex A. Ces acides aminés ont été choisis à cause qu'ils sont conservés parmi les glycosidases de la famille #20 dont un des membres a été crystalisé récemment. Ces études suggèrent que E323 ainsi que E462 sont localisés dans un site actif de l'enzyme Hex A tandis que E323 est un acide aminé catalytique.

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ABBREVIATIONS AND SYMBOLS

βgal :	beta-galactosidase
bp :	base pair
cDNA :	complementary DNA
Cer :	ceramide
CHS :	chitobiase from *Serratia marcescens*
cm^2:	centimeters squared
CMV:	cytomegalovirus
CNS :	central nervous system
cRNA :	synthetic transcript of DNA
del:	deletion
DNA:	deoxyribonucleic acid
ECL:	enhanced chemiluminescence
ER or RER:	endoplasmic reticulum
FBS:	fetal bovine serum
fg:	femtogram
G_{A2}:	asialo version of G_{M2} ganglioside
GAD:	G_{M2} activator deficiency
Gal:	galactose
GalNAc:	N-acetylgalactosamine
Glc:	glucose
GlcNAc:	N-acetylglucosamine
GM2A:	the gene coding for the G_{M2} activator protein
h:	hours
Hex:	hexosaminidase
HEXA:	the gene encoding the human α-subunit of the isoenzyme Hex A

HEXB:	the gene encoding the human β-subunit of the isoenzyme Hex B
Hexa:	the gene encoding the mouse α-subunit of the isoenzyme Hex A
Hexb:	the gene encoding the mouse β-subunit of the isoenzyme Hex B
Hex A:	the hexosaminidase isoenzyme A
Hex B:	the hexosaminidase isoenzyme B
[^{3}H]-1-ATB-GalNAc:	3 azi-1-[([6-^{3}H]-2-acetamido-2-deoxy-1-β-D-galactopyranosyl)thio]-butane
Ig:	immunoglobulin
kb:	kilobase
kD or kDa:	kilodalton
Km:	the substrate concentration at which the rate of a reaction is one-half maximal
M:	molarity, moles/liter
MCB:	membranous cytoplasmic bodies
MEM:	minimal essential medium
mg:	milligram
min:	minute
mM:	millimolar
ml:	milliliter
mRNA:	messenger RNA
msec:	millisecond
4MUG:	4-methylumbelliferyl-β-D-N-acetylglucosaminide
4MUGS:	4-methylumbelliferyl-β-D-N-acetylglucosamine-6-sulfate
NG:	neuroglial
nm:	nanometer
nmol:	nanomole

OD:	optical density
oligo:	oligonucleotide
pI:	isoelectric point
pH:	- log $[H^+]$
PCR:	polymerase chain reaction
pg:	picogram
PBS:	phosphate buffered saline
RNA:	ribonucleic acid
RTPCR:	reverse transcriptase followed by PCR
SD:	Sandhoff disease
snRNP:	small nuclear ribonucleoprotein
ss:	splice site
SSCP:	single stranded conformation polymorphism
SV40:	simian virus 40
TSD:	Tay-Sachs disease
uFD:	microFarad
ug:	microgram
ul:	microliter
V:	volts
Vmax:	the maximal velocity of a reaction

CHAPTER 1

INTRODUCTION

INTRODUCTION

1.1 Lysosomal storage disorders and Tay-Sachs Disease

Lysosomal disorders result from the deficiency of a lysosomal hydrolase leading to the gradual accumulation of the corresponding substrate (Thompson et al, 1991). They all exhibit clinical and allelic heterogeneity and are recessively inherited. Clinical heterogeneity is usually caused by small variations in the amount of residual enzyme activity due to heteroallelism. Furthermore, mutations at different gene loci may confer similar clinical and biochemical phenotypes.

Tay-Sachs disease (TSD) results from a deficiency of the lysosomal enzyme β-N-acetyl-hexosaminidase A (Hex A) (Okada and O'Brien, 1969) leading to the pathological accumulation in neuronal cells of G_{M2} ganglioside, an acidic glycolipid terminating in a β-D-N-acetylgalactosaminyl linkage (Svennerholm, 1962). Small differences in residual Hex A activity, due to a wide range of mutations at the *HEXA* locus, result in varying clinical manifestations, from the infantile acute phenotype to chronic forms of the disorder (Conzelmann and Sandhoff, 1983; Leinekugel et al, 1992; Mahuran, 1991). The infantile form of TSD and that of two other related but genetically distinct disorders, Sandhoff disease (SD) and G_{M2} activator deficiency (GAD), are neurologically indistinguishable. The products of the gene loci responsible for these three disorders are all required for the hydrolysis of G_{M2} ganglioside. Therefore, complete absence of any one of them will produce an identical pattern of G_{M2} ganglioside

accumulation and similar clinical manifestations. Collectively these three disorders are referred to as the G_{M2} gangliosidosis (Suzuki and Chen, 1967).

1. 2 Historical introduction

The clinical characteristics of TSD were first described by an ophthalmologist Warren Tay (Tay, 1881). He described, as the most prominent ocular feature, macular palor contrasted with prominence of the macular fovea centralis. The most prominent histological feature, the distended cytoplasm of neurons, was later reported by the neurologist Bernard Sachs (Sachs, 1887). Sachs, who was the first to recognize the high prevalence of the disease in the Jewish population, gave to the condition the name late infantile amaurotic family idiocy (Sachs, 1896). The initial contribution of Drs. Tay and Sachs to the delineation of this disorder eventually gave it the name Tay-Sachs disease.

The storage material in TSD patients was initially identified by Ernst Klenk, who gave the name of gangliosides to the new group of sialic acid containing glycolipids found in the brain (Klenk, 1939; Klenk 1941). The main neuronal storage compound, G_{M2} ganglioside, was identified in 1962 by Svennerholm and its structure later elucidated by Ledeen and Salsman (1965). In 1968 Robinson and Stirling made a key discovery that would later solve the TSD paradox; why, if the hydrolysis of G_{M2} ganglioside involves the removal of a β-D-N-acetylhexosamine residue, do TSD patients have normal or slightly elevated hexosaminidase levels? Robinson and Stirling, using electrophoresis, separated two forms of hexosaminidase, a heat-labile (Hex A) and a heat-stable (Hex B)

form. In 1969 Okada and O'Brien and Sandhoff identified the enzymatic defect in TSD as a deficiency of the heat-labile form of hexosaminidase (Hex A). Hexosaminidase activity in TSD patients is, therefore, due to Hex B. The ability to test specifically for a deficiency of Hex A, due to its heat-lability, paved the way for carrier screening programs and prenatal diagnosis in at-risk pregnancies (Kaback, 1972). Sandhoff observed that some patients diagnosed with TSD lacked both Hex A and Hex B activity (Sandhoff et al, 1968; Sandhoff et al 1971). These observations were able to be interpreted once the subunit structures of Hex A and B were determined (Srivastava and Beutler, 1973; Mahuran and Lowden, 1980). Hex A is composed of an α - (~55 kD) and β -(~20-30 kD) subunit while Hex B is a homodimer of β-subunits. A deficiency of β-subunits leads to the absence of both Hex A and Hex B causing Sandhoff disease. In these patients a third hexosaminidase isoenzyme, Hex S ($\alpha\alpha$), was shown to be present (Sandhoff, 1969, Sandhoff et al, 1971; Ikonne et al, 1975). It is believed not to play a significant physiological role (Mahuran et al, 1985). A deficiency of the α-subunit causes TSD. G_{M2} gangliosidosis patients with normal Hex A and B activities were later found to have a deficiency of a third gene product known as the G_{M2} activator protein (Conzelmann and Sandhoff, 1978; Hechtman et al, 1982; Hirabayashi et al, 1983).

In recent years (1980's- 1990's) knowledge of the proteolytic processing (Mahuran, 1995) of the α- and β-subunits has been refined and the loci encoding these subunits, *HEXA* and *HEXB*, mapped to chromosomes 15q23-> q24 (Gilbert et al, 1975; Takeda et al, 1990)

and 5q13 (Fox et al, 1984) respectively. The genes have been cloned (Myerowitz et al, 1985; Neote et al, 1988) their cDNA's sequenced and the intron-exon boundaries of their genomic loci determined (Proia and Soravia, 1987; Proia, 1988). These developments have permitted analysis of mutations resulting in the identification to date of over 54 *HEXA* and 12 *HEXB* mutations (Gravel et al, 1993; Hechtman and Kaplan, 1993). The implication of many *HEXA* mutations in the TSD phenotype has been confirmed by expression analysis. The COS system is the most commonly used among several systems that have been established for the expression of *HEXA* alleles. Expression studies have been used to distinguish benign from deleterious mutations as well as to identify functionally important residues using site-directed mutagenesis (Brown and Mahuran, 1993).

To date, there is no cure for TSD. Attempts at therapy have been largely unsuccessful. The major obstacle to the delivery of enzyme to the affected tissue is the blood-brain barrier (Von Specht et al, 1979; Krivit and Paul, 1986). Recent strategies have been proposed involving the use of i) viral vectors carrying expressible cDNA's (Friden et al, 1993) and ii) fusion proteins where the peptide used has a high affinity for neuronal cells (Palella et al, 1989; Freese et al, 1990; Huang et al 1992). Testing of these experimental strategies requires animal models of TSD. Several animals (cats, dogs and Yorkshire swine) suffering from G_{M2} gangliosidoses were identified in the late 1970's (Cork et al, 1977; Gambetti et al, 1970; Eto et al, 1984; Kosanke et al, 1978) and recently, Sango (1995) and Phaneuf (in press), used gene targeting in embryonic stem cells to establish

mouse "knockout" models for TSD and SD (Yamanaka et al, 1994; Taniike et al, 1995; Phaneuf et al, in press).

1.3 Clinical phenotypes and pathology of TSD

1.3.1 Clinical phenotypes

The severity of the neurological phenotype is inversely related to residual Hex A activity in patient's neurons. The analysis of enzyme activity in individuals carrying benign or pseudodeficient *HEXA* mutations indicates that residual hexosaminidase A activity of only 10-20% of normal is necessary for a normal phenotype (Conzelmann and Sandhoff, 1983; Leinekugel et al, 1992). Therefore the different clinical phenotypes occurring in G_{M2} gangliosidosis patients are likely to be caused by very small variations in the residual amount of enzyme activity.

Initially, it was proposed that TSD patients be classified according to the age of onset of the phenotype. However this classification system is inconsistent due to the early onset of symptoms (in childhood) in some patients with the adult form of the disorder. An alternative is to classify patients using the dominance of encephalopathy as the primary clinical delineator. This classification not only refers to TSD but also to SD and GAD. Patients are classified in either of the following categories i) infantile acute G_{M2} gangliosidoses (same as the classical infantile form), ii) subacute G_{M2} gangliosidoses which refers to patients with the late- infantile or juvenile forms that are fatal in

childhood or early adulthood or iii) chronic G_{M2} gangliosidoses which refers to juvenile and adult-onset patients whose phenotype is compatible with long survival (Gravel, 1993). The pseudodeficient genotypes however, do not fit into any of the above categories since they confer a normal phenotype. These individuals' hexosaminidase is able to hydrolyze G_{M2} ganglioside in the low-normal range but is defective toward the artificial substrates used in the screening (enzyme) test. These phenotypically normal individuals are therefore misclassified as carriers by the enzyme test.

In the infantile acute form the symptoms, which include mild motor weakness and an exaggerated startle reaction to sharp sounds, begin at 3 to 5 months of age. These infants have difficulty in attaining gross motor skills such as crawling and sitting unsupported. A more definite diagnosis of TSD is usually obtained after an ophthalmoscopic examination. A cherry-red spot is clearly identified in the retina due to macular pallor and prominence of the fovea centralis. After 10 months of age the disease progresses rapidly, vision deteriorates, the patient becomes less responsive to the surroundings and starts to suffer from seizures. Macrocephaly becomes apparent at 18 months of age. Patients usually die between the ages of 2 and 4 at which time they have reached a vegetative state. The cause of death is usually bronchopneumonia. The infantile form of SD which presents with these same symptoms can be distinguished from TSD by the presence of nonneurological symptoms, such as hepatosplenomegaly and skeletal abnormalities (Gravel, 1995).

Patients suffering from the subacute form of TSD experience their initial symptoms between the ages of 2 and 10. Unlike the infantile form, these patients do not show any macular degeneration even though they lose their vision in the later stages of the disease. They do suffer from ataxia and incoordination, developmental regression, dementia, increasing frequency of seizures and spasticity. A vegetative state is reached at 10 to 15 years of age followed by death, the cause of which is usually intercurrent infection (Suzuki et al, 1970; Menkes et al, 1971; Brett et al, 1973; MacLeod et al, 1977; Suzuki and Vanier, 1991).

The clinical phenotype for the chronic form of the disorder is more difficult to define since there are several presentations and there is a wide variability of symptoms (Navon et al, 1986; Frederico et al, 1991). In some families phenotypic heterogeneity is observed even between siblings. There are, however, some recurring neurological features common to most chronic G_{M2} gangliosidosis patients. Progressive dystonia, muscle wasting and weakness (Parnes et al, 1985; Johnson et al, 1982) are common in the late-onset variants of the G_{M2} gangliosidoses as is psychosis (Argov and Navon, 1984; Lichtenberg et al, 1988; Streifler et al, 1989). Dementia is not a prominent feature and neither is loss of vision, seen in the more acute forms of G_{M2} gangliosidoses. Due to the diversity in clinical symptoms associated with the chronic form of TSD, patients are commonly misdiagnosed as having other neurological conditions. These include, atypical spinocerebellar degeneration, spinal muscular atrophy, atypical Friedreich ataxia (with sensory modalities intact), Kugelberg-Welander disease and amyotrophic lateral sclerosis

(Rapin et al, 1976; Yaffe et al, 1979; Argov and Navon, 1984). The possibility that juvenile spinal muscular atrophy may be caused by *GM2A* or *HEXB* mutations was ruled out once the gene causing the phenotype was localized to 5q11.2-13.3 (Kleyn et al, 1991, Heng et al, 1993; Swallow et al, 1993).

1.3.2 Pathology

The most prominent histopathological feature in infantile TSD patients is the presence of swollen neurons whose nuclei and Nissl substance are pushed to the periphery. The swelling of neurons is due to the pathological accumulation of storage material which is associated with granules having strong acid phosphatase activity (Lazarus et al, 1962). The major storage compound is G_{M2} ganglioside (Jatzkewitz et al, 1965, Sandhoff et al, 1971). The storage material appears, under electron microscopy, in the form of membranous cytoplasmic bodies (MCB's) which are concentrically arranged lamellar structures composed of precipitated membrane components and accumulating lipids (Terry and Weiss, 1963). Meganeurites are also quite prominent and have MCB's (Purpura and Suzuki, 1976).

The pathology in the chronic form of TSD, in conformity with the phenotypic variability, shows a variety of clinicopathological features (Argov and Navon, 1984). The most common features are a decrease in brain weight, neuronal swelling and mild to moderate cerebral atrophy. Unlike the infantile form, some or all the storage material in the CNS has staining characteristics similar to the storage material in ceroid lipofuscinosis.

Furthermore, glycolipid storage is restricted to specific brain regions. The cortex appears normal whereas the hippocampus, brain stem nuclei, the spinal cord and retina are markedly affected (Suzuki et al, 1970; Rapin et al 1976; Jellinger et al, 1982).

1. 4 Incidence and population genetics

TSD has an increased carrier frequency in several ethnic groups, namely, the Ashkenazi Jewish population (1/31) (Kaback et al, 1993), the French-Canadian population of Eastern Quebec (1/28) (Andermann, et al 1973), the Japanese (Murakami, 1957), the Pennsylvania Dutch (Kelly et al, 1975) and the Cajuns from Louisianna (McDowell et al, 1992). Each population seems to have its own particular mutation spectrum. The three most common mutations in the Ashkenazi Jewish population are, in descending order of frequency: +TATC1278, IVS12, +1 G->C and G805A (Triggs-Raine et al, 1990; Paw et al, 1990a; Grebner and Tomczak, 1991; Landels et al, 1991; Fernandes et al, 1992a). In contrast, the most common mutation among French-Canadians is a 7.6 kb deletion at the 5' end of the gene (Myerowitz and Hogikyan, 1986; Myerowitz and Hogikyan, 1987; Hechtman et al, 1990) followed by a IVS 7, +1 G->A in the Lac St-Jean area (Hechtman et al, 1992). Identification of population-specific alleles is essential for any community-based TSD carrier screening program that incorporates DNA testing for the diagnosis of genotypes.

The enzyme test for determination of phenotype is insensitive to the underlying allelic heterogeneity at the *HEXA* locus and will therefore not distinguish between hetero-

zygotes for benign and disease causing mutations (Kaback, unpublished results). Consequently, carrier frequencies, for disease-related alleles, based on enzymatically-defined carriers may not reflect the true carrier frequency. In the case of the Ashkenazi-Jewish community, only 2% of enzyme-defined carriers have a pseudodeficiency (benign) mutation which affects only marginally the true carrier frequency in this group. In contrast, when a similar analysis is done in non-Jewish populations the outcome is more dramatic, since the mutation spectrum is radically different. In a cohort of 34,532 non-Jewish individuals, tested by the California Tay-Sachs Disease Prevention Program, the enzyme-based carrier rate is 1 in 167 (Kaback et al, 1993). Approximately 35% of enzymatically defined carriers, however, are heterozygotes for a pseudodeficiency allele (Triggs-Raine et al, 1992) reducing the carrier rate for disease-related alleles to 1 in 256.

The reason(s) for the elevated frequency of deleterious *HEXA* variants in certain populations, despite their elimination through non-reproducing homozygotes, are still unknown. Two hypotheses have been proposed. The first suggests that founder effect or genetic drift, could account for the high frequency of these alleles (Chase and McKusick, 1972; Chase, 1977). The second hypothesis suggests that heterozygotes may have a selective advantage relative to homozygous normal individuals which permitted survival or enhanced reproduction in the presence of some environmental factor. The selection hypothesis is favoured by the finding of heteroallelism within high risk populations. Myrianthopoulos (Myrianthopoulos et al, 1966; Myrianthopoulos et al, 1977) suggested that the selective force may have been resistance to pulmonary tuberculosis. Kaback et

al (1993) speculated that a subtle alteration in the sphingolipid composition of the cell membrane could affect the susceptibility to pathogens via host-defense mechanisms that act through cellular responses. Heterozygote advantage increases the gene frequency since TSD heterozygotes are maintained in the population. On the other hand, selection against homozygotes does not appreciably affect gene frequency. It is probable that the factor(s) reponsible for the increase in gene frequency are no longer operative but their effects are still apparent.

Neither hypothesis has been proven and indeed the two ideas may not be mutually exclusive. Whatever the case, screening for TSD carriers will not decrease the gene frequency, in fact, it has a tendency to maintain the mutant genes in the population. In contrast, its effect on the homozygote frequency has been dramatic. The impact of screening on the incidence of TSD in the Ashkenazi Jewish population of the United States and Canada, from 1970 to 1993, was to decrease it by over 90% (Kaback et al, 1993). On the other hand, the TSD disease incidence in the non-Ashkenazi Jewish population has not changed emphasizing the effectiveness of TSD screening.

1. 5 The biochemical defect

1.5.1 G_{M2} ganglioside

"Ganglioside" is a generic term for glycosphingolipids that contain sialic acids (Svennerholm, 1980). G_{M2} ganglioside is composed of a hydrophobic ceramide (N-

acylsphingosine) and an oligosaccharide chain that bears one sialic acid (N-acetyl-neuraminic acid) molecule (Fig 1). The hydrolysis of G_{M2} ganglioside, which occurs in the lysosome, involves the removal of the terminal β-N-acetyl-galactosaminyl residue by Hex A.

Gangliosides are localized in the outer leaflet of the plasma membrane of all animal cells. It is envisaged that the ceramide moiety acts as an anchor in the cell membrane exposing the hydrophilic portion to the extracellular space (Thompson and Tillack, 1985). No key physiological role has been unequivocally demonstrated for gangliosides. However, it has been suggested that they may act as receptors on the cell surface and as regulators and modulators of membrane machinery (Ando, 1983). They are most probably involved in cell-cell interactions and cell differentiation (Hakomori, 1984; Roseman, 1985; Varki et al, 1991). Some gangliosides have been implicated as binding sites for bacterial toxins, viruses, coreceptors for hormones, in cell mobility and in synaptic transmission (Akawa and Nagai, 1978; Svennerholm, 1984; Hanai et al, 1988; Kojima and Hakomori, 1991).

The highest ganglioside content is found in the gray matter of the brain (Svennerholm, 1980). Since G_{M2} ganglioside is found exclusively in the brain (Ando, 1983), TSD manifests itself as a disorder of the central nervous sytem.

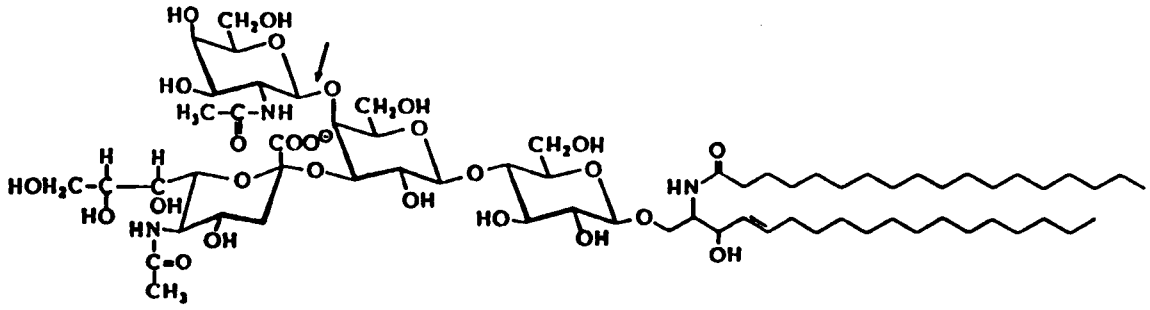

Figure 1: The structure of G_{M2} ganglioside.

1.5.2 The hexosaminidase isoenzymes

Hexosaminidases are exohydrolases that hydrolyze the β-glycoside bond of two amino sugars, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine (Robinson and Stirling, 1968; Robinson et al, 1972). The two major hexosaminidase isoenzymes in humans are Hex A and Hex B. Hex A is composed of an α– and β-subunit and Hex B is a homodimer of β-subunits (Mahuran and Lowden, 1980). These isoenzymes differ in pI with Hex A (pI=4.9) being more acidic than Hex B (pI=6.9). Deficient or defective α-subunit causes TSD in which Hex A is absent. Absent or defective β-subunit causes SD which is marked by absence of both Hex A and Hex B. Hex S, an isoenzyme found only in SD patients, is a homodimer of α–subunits and is more acidic and heat-labile than Hex A (Ikonne et al, 1975, Beutler et al, 1975; Geiger et al, 1977). Other minor species of hexosaminidases include the Hex I's, Hex P and Hex C (reviewed in Mahuran et al, 1985). The Hex I's are a heterogeneous group of hexosaminidases with pIs intermediate between Hex A and B . These species are named according to the order in which they elute from an anion-exchange column. Thus Hex I1 elutes following HexB and is followed by Hex I2. Hex I's are forms of Hex B or Hex A in various stages of maturation. Thus, serum Hex I's are presumed to be underphosphorylated derivatives of Hex B and therefore to differ in sialic acid content. Placental Hex I2 is composed of partially processed α- and β–polypeptides. Hex I's are found in serum and urine and their abundance varies between tissues. In human placenta, Hex I's make up approximately 3 % of hexosaminidase activity. Hex P, a Hex I-like form with a pI of 6.3 to 6.7, is found

in large quantities in the serum of pregnant women (Stirling, 1972, Geiger et al, 1978). Hex C was initially confused with Hex S since the two enzymes have similar electrophoretic mobility (Hooghwinkel et al, 1972). Hex C is of cytoplasmic origin and is antigenically unrelated to the other hexosaminidase isoenzymes (Beutler and Kuhl, 1977).

The Hex A and B isozymes hydrolyze a broad spectrum of substrates which have a β-GlcNAc or β-GalNAc terminal non-reducing sugar (Mahuran et al, 1985). The α- and β-subunit active sites have different substrate specificities (Kytzia and Sandhoff, 1985). Both hydrolyze neutral water soluble substrates but only the former hydrolyzes negatively charged substrates (Kresse et al, 1981). G_{M2} ganglioside is negatively charged and can only be hydrolyzed at the α-subunit active site of Hex A. Other negatively charged substrates hydrolyzed uniquely by Hex A include polysaccharides with a penultimate, charged (uronic) residue (chondroitin or dermatan sulfate) (Bearpark and Stirling, 1978) or those with a negative charge on the terminal N-acetyl-glucosamine residue (keratan sulfate) (Kresse et al, 1981; Kytzia et al, 1984).

Synthetic substrates have proven useful for routine assay of hexosaminidases since the structure of the glycoside aglycone seems not to influence the rate of their hydrolysis (Mahuran et al, 1985) . The most sensitive and most commonly used substrates are the fluorogenic compounds 4MUG, 4-methylumbelliferyl-GlcNAc, (Leaback and Walker, 1961) and 4MUGS, 4-methylumbelliferyl-GlcNAc-6-SO_4 (Bayleran et al, 1984; Ben-

Yoseph et al, 1985; Charrow et al, 1985). The aglycone, 4-methylumbelliferone, fluoresces following substrate hydrolysis providing a rapid, sensitive and accurate means of measuring hexosaminidase activity. 4MUG is hydrolyzed by both Hex A and B but 4MUGS (which is negatively charged) only by the α-subunit active site of Hex A. These substrates have not only facilitated studies of the properties of the hexosaminidase isozymes but have also simplified the diagnosis of TSD carriers and patients (Fuchs et al, 1983; Ben-Yoseph et al, 1985; Kytzia and Sandhoff, 1985; Bayleran et al, 1987). The biochemical properties of Hex A, Hex B and Hex S are summarized in figure 2 (Kytzia and Sandhoff, 1985).

The major difference between the action of Hex A toward negatively-charged hydrophilic and hydrophobic (G_{M2} ganglioside) substrates is the requirement for presence of the G_{M2} activator protein for hydrolysis of the latter group of substrates (Li et al, 1973; Hechtman, 1977; Conzelmann and Sandhoff, 1979). The activator protein solubilizes G_{M2} ganglioside from the lysosomal membrane and presents a substrate monomer to Hex A (Conzelmann et al, 1982). Conzelmann et al (1982) showed that the activator binds G_{A2} and G_{M3} less efficiently than G_{M2} ganglioside. Therefore the sugar and sialic acid residues of G_{M2} ganglioside seem to be important for recognition. The strong binding of activator to octyl-Sepharose suggests that it also recognizes the large hydrophobic portion of G_{M2} ganglioside. Since activator does not promote glycolipid breakdown by Hex B it is thought that protein-protein recognition is essential for activator-dependent G_{M2} ganglioside hydrolysis.

K_m and V_{max} values of Hex isoenzymes toward artificial substrates

Enzyme	4MUG		4MUGS	
	K_m	V_{max}	K_m	V_{max}
Hex B	0.91	4.4×10^{-4}	3.40	1.2×10^{-4}
Hex A	0.90	1.8×10^{-4}	0.31	2.5×10^{-4}
Hex S	2.80	1.5×10^{-4}	0.33	5.6×10^{-4}

Figure 2: The K_m (in mM) and V_{max} (in mol/min/mg) values of human lysosomal β-hexosaminidases toward the 4MUG and 4MUGS substrates. (From Kytzia and Sandhoff, 1985)

The difference in substrate specificity between Hex A and B determines the nature of the storage compounds in TSD and SD patients (Svennerholm, 1962). The major neuronal storage compound in both variants is G_{M2} ganglioside. However, since SD patients lack both Hex A and B globoside ((GalNAc(β1->3) Gal(α1->4) Gal(β1->4)Glc(β1->1^1)Cer) accumulation also occurs in their visceral organs (Snyder et al, 1972; Dolman et al, 1973; Hadfield et al, 1977). The presence of Hex B, which hydrolyzes this neutral substrate, permits TSD patients to bypass this metabolic block. SD can also be distinguished from TSD by higher storage levels of glycolipid G_{A2}, the asialo derivative of G_{M2} ganglioside, in the brain (Suzuki et al, 1971). The lower accumulation of G_{A2} in TSD may be caused by a slow rate of hydrolysis of the α-sialyl linkage of G_{M2} ganglioside by a ganglioside sialidase followed by Hex B hydrolysis of the neutral product G_{A2} glycolipid (Conzelmann and Sandhoff, 1979).

Hexosaminidases are found at low levels in all tissues consistent with their role as house-keeping enzymes. Recent studies in mice revealed, however, that there is differential expression of *Hexa* and *Hexb* mRNA in different tissues, most markedly in the testis (Yamanaka et al, 1994; Triggs-Raine et al; 1994). Hexa mRNA is more abundantly expressed in mouse testis, in comparison to low levels of Hexb mRNA, probably resulting in different levels of Hex A, B and S in this tissue. Two roles for hexosaminidases in fertilization have been proposed: (i) the high levels of hexosaminidase activity in the sperm acrosome may facilitate its penetration through the zona pellucida (Miller et al, 1993) and (ii) the release of hexosaminidase by eggs at

fertilization is thought to prevent polyspermy by inactivating sperm receptors (Miller et al, 1992).

1.6 The processing and targeting of hexosaminidases

The α– and β-polypeptides undergo several processing events prior to being targeted to the lysosome (reviewed in Kornfeld and Kornfeld, 1985). The nascent polypeptides, the pre-pro-α or β subunit, undergo the cotranslational modifications characteristic of the secreted and plasma membrane proteins (Von Heijne, 1990) giving rise to the pro-α (67 kD) or β (63 kD) polypeptides. These reactions include i) the removal of the hydrophobic, N- terminal 'signal' peptide (the 'pre-' sequence) (Fig 3) and ii) the glycosylation of selected asparagine residues by a dolichol intermediate. All three putative glycosylation sites in the α-subunit are glycosylated in cultured human fibroblasts whereas four of five potential sites are glycosylated in the β-subunit (O'Dowd et al, 1988; Sonderfeld- Fresko and Proia, 1989; Weitz and Proia, 1992). A phosphotransferase catalyzes the phosphorylation of the glycosylated proenzyme by transferring a Glc-NAc-1-phosphate to selected mannose residues forming a phosphodiester intermediate (Reitman and Kornfeld, 1981; Waheed et al, 1982). Proteins that fail to acquire phosphomannosyl residues are secreted. Proia et al (1984) demonstrated that the phosphorylation event, which occurs in the late ER, precedes dimerization of the α- and β-subunits. Phosphorylated enzymes are targeted to the lysosome in the late ER via the mannose-6-phosphate receptor pathway (Erickson et al,

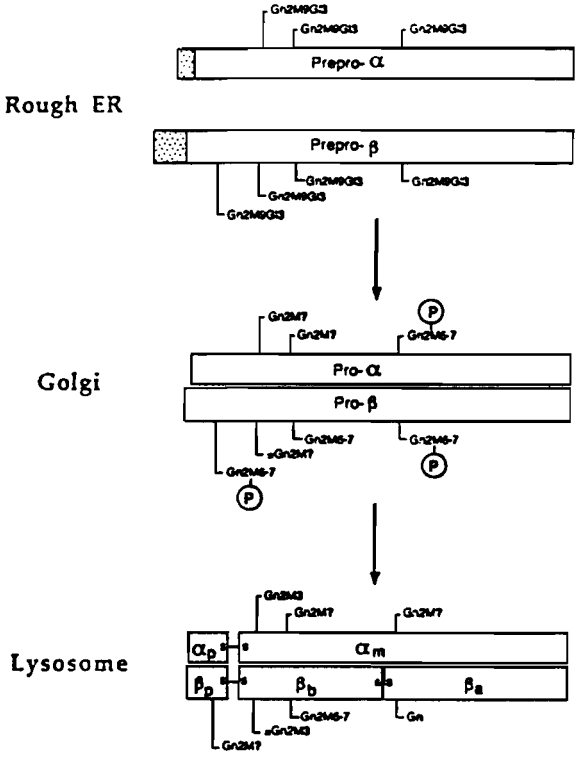

Figure 3: The processing of the α- and β-subunits of Hex A. The cellular compartments in which processing takes place are indicated on the left. The oligosaccharide attachments are shown, the circled P represents phosphorylation and the stippled boxes correspond to signal peptides. (From Gravel et al, 1995)

1981). The enzyme N-acetyl-glucosamine-1-phosphodiester-α-N-acetyl-glucosaminidase removes the terminal GlcNAc from the phosphomannosyl moiety exposing the phosphomannosyl recognition marker (Varki and Kornfeld, 1980). This is followed by binding, in the trans-Golgi network, of the phosphorylated enzyme to the mannose phosphate receptor. The protein-receptor complex reaches the lysosome by a pathway not yet completely understood (Croze et al, 1989). In a prelysosomal compartment the enzyme is released into the lumen as a consequence of acidification and released receptors are recycled back to the Golgi apparatus.

Once in the lysosome, the hexosaminidase polypeptides undergo the final stages of maturation which involve extensive proteolytic modifications (Little et al, 1988, Quon et al, 1989). These modifications are very specific, do not alter the activity of hexosaminidase and involve mostly a loss of basic amino acids. The mature forms of the α– and β-polypeptides, which are more acidic than their pro-subunit counterparts, consist of an N terminal α_p fragment (52 amino acids), a C-terminal α_m fragment (440 or 441 amino acids), and three sub-fragments of the β-subunit, β_p (58 amino acids), β_b (190 amino acids) and β_a (242 amino acids) (O'Dowd et al, 1988; Hubbes et al 1989). The unique proteolytic cleavage that occurs in the pro-β chain to produce β_a and β_b involves the removal of residues, Arg312- Gln-Asn-Lys, that are located in a hydrophilic disulfide loop structure between Cys306 and Cys360. The functional implication of this proteolytic event is unknown. However, it has been demonstrated that these residues are

not involved in the stability of the native isoenzyme, intracellular targeting or substrate specificity. The residue(s) may be important for early processing events such as folding of the β-subunit and disulfide bond formation (Sagherian et al, 1993). A small fraction of the pro-Hex A and pro-Hex B are secreted and make up the pool of serum hexosaminidase (Kornfeld, 1990).

1.7 Organization and evolutionary relationships of the human *HEXA* and *HEXB* genes

The elucidation of the primary amino acid sequences of the α– and β-subunits and the isolation of human *HEXA* messenger by polysome immunoselection were the first steps in the isolation and cloning of the *HEXA* (Myerowitz and Proia, 1984; Myerowitz et al, 1985; Korneluk et al 1986) and *HEXB* (O'Dowd et al 1985) genes. Both the *HEXA* and *HEXB* genes span 14 exons and 13 introns but differ in length; 35 kb and 45 kb respectively. The degree of homology of their amino acid sequences is 57% and the exon-intron boundaries of the two genes are identical except for the first exon (Proia and Soravia, 1987; Proia, 1988; Neote et al, 1988). This suggests that the genes are evolutionarily related and may have evolved from the duplication of an ancestral gene. Despite the striking degree of homology there is considerable divergence of amino acid sequence at the amino terminal end. It has been proposed that the amino terminal end may have structural elements responsible for the difference in substrate specificity between the α– and β-subunit active sites.

The organization of the *HEXA* and *B* genes is similar except for the expanded intron 1 of the *HEXA* gene. The 5' introns are larger than the 3' and the exons show a strict size distribution typical of eukaryotic genes (Blake, 1983). The first and last exons in the *HEXA* gene contain untranslated sequence, the first encoding both the signal sequence and the amino-terminal extension (Proia and Soravia, 1987). This contrasts with the organization in many secretory and membrane proteins where the signal sequence is carried on a separate exon (Gilbert , 1985).

The *HEXA* promoter has not been as extensively characterized as that of *HEXB* and the proposed α-subunit promoter sequences have not yet been confirmed in expression experiments. However promoter-like sequences have been identified 5' to the *HEXA* gene, namely, a TATA and CAAT box motif within a GC-rich region (Proia and Soravia, 1987). The *HEXB* gene promoter has four GC boxes, a CAAT box motif, two sequences related to the AP-1 binding site but lacks a TATA box (Neote et al, 1988). The exact position of transcription initiation remains to be determined. It is possible that there are several transcription start sites since this is typical of promoters lacking a TATA box. A cis-acting DNA element that responds to steroid hormone-receptor complexes was also identified in the *HEXB* promoter region. It is very similar to the sequence of the progesterone response element found in the chick lysozyme gene. The functional role this element plays in the expression of the *HEXB* gene remains to be determined. The similiarity of the hexosaminidase promoters to those of other housekeeping genes explains the ubiquitous presence of β-hexosaminidase. It is of note that the human

HEXA gene has, in addition to the GC-rich region characteristic of housekeeping genes, a TATA box which is more typical of tissue-specific genes.

The mouse *Hexa* and *Hexb* genes have also been cloned (Bapat et al, 1988; Beccari et al, 1992; Yamanaka et al, 1994; Triggs-Raine et al, 1994; Wakamatsu et al, 1994). There is extensive conservation between the human and mouse hexosaminidase genes. The intron/exon junctions as well as the amino acid sequence have been highly preserved. The cloning of the mouse genes has enabled the creation of mouse "knockout" models for Tay-Sachs and Sandhoff diseases which may be useful for investigation of therapeutic strategies (Yamanaka et al, 1994; Taniike et al, 1995; Phaneuf et al, in press). Remarkably, the TSD mouse, which displays <1 % of normal β-hexosaminidase activity and an accumulation of G_{M2} ganglioside in the nervous system, does not appear to have the TSD phenotype. In contrast, the SD mouse not only exhibits a deficiency in hexosaminidase activity but also the disease phenotype.

1. 8 Mutations at the HEXA gene

Seventy-four mutations have been identified in the *HEXA* gene to date. The majority of these are missense, followed by: mutations which cause splicing, nonsense and frameshift mutations. Approximately 40% of *HEXA* mutations occur at CpG dinucleotide sites. The greatest number of mutations are found in exons 1, 5 to 8 and 14. Among mutations identified in G_{M2} gangliosidosis patients the majority cause the infantile form of TSD. Approximately 19% cause the subacute form, two are responsible for the chronic form

(G269S, K197T) (Paw et al, 1989; Navon and Proia, 1989; Akli et al, 1993) and three others associated with the pseudodeficiency phenotype (R247W, R249W, R249Q) (Triggs-Raine et al, 1992; Cao et al, 1993, Callahan et al, 1995).

All *HEXA* genotypes causing the infantile form of TSD are associated with complete absence of enzymatic activity. In contrast, it is sometimes difficult to establish genotype-phenotype correlations for the subacute and chronic forms of TSD. In general, alleles associated with milder phenotypes are dominant to those causing more severe forms of the disease when two different alleles occur in a compound heterozygous individual. The R178H mutation appears to be an exception to this rule since homozygotes for this mutation suffer from the subacute form of TSD whereas compound heterozygotes harboring a null allele in addition to R178H have the infantile form of the disease (Tanaka et al, 1990).

Mutations causing subtle changes of enzymic properties may be informative as to the role of the affected residue in the maturation and/or function of the polypeptide. Two such mutations have been proposed to affect residues that are components of the α-subunit active site; R178H (Ohno and Suzuki, 1988) and D258H (Fernandes et al, 1992b). Expression analysis of the R178H (Brown and Mahuran, 1993) and the analysis of patient fibroblast Hex A carrying the D258H (Bayleran et al, 1987) substitution suggest that these residues may be involved in α-subunit catalysis (Kytzia et al, 1983). Indeed, these two mutations are classified as B1 mutations, a designation which is reserved for a

biochemical phenotype in which Hex A is unable to hydrolyze α-subunit-specific substrates, 4MUGS or G_{M2} ganglioside, but appears to fold and dimerize normally.

1.9 TSD screening

Since there is no treatment for this disorder, TSD prevention programs have focused on identifying couples at risk for conceiving an affected homozygote. This goal is achieved through carrier screening which detects phenotypically normal individuals who are heterozygous for a TSD gene.

In the 1970's TSD carrier screening in the Ashkenazi Jewish population was initiated at the community level (Kaback and Zeiger, 1972). This was the first multiphasic voluntary effort for the prevention of a genetic disease. The program included public education, carrier detection, genetic counselling and prenatal diagnosis. Others followed soon after with similar programs (Canada, Israel, South Africa, Australia, South America and Europe) (Kaback et al, 1993).

The enzyme test for the determination of TSD genotypes involves a thermal fractionation assay that selectively inactivates Hex A (O'Brien et al, 1970; Dance et al, 1970; Kaback, 1972). The remaining Hex activity (Hex B) is subtracted from the activity in unheated samples (total hex) to determine the % Hex A. Hex assays are routinely performed with 4MUG as substrate and many centers use automated methods to measure the two isozymes in serum.

The inherent limitations of the enzyme test include misclassification of individuals which may occur for several reasons. All quantitative tests used to determine genotypes generate probability density functions for carriers and normal values in which a region of overlap or 'gray zone' occurs. Individuals with test results within this region cannot be given a definitive classification.

In addition, pregnant women have a high level of Hex P, an isoenzyme similar to Hex B, whose presence causes false positive results, i.e. misclassification of homozygous normal women as carriers. This problem is usually dealt with by performing the enzyme test on leukocyte samples (Kaback, 1977).

Another pitfall of the enzyme assay is its inability to correctly identify genotypes in individuals carrying certain allelic *HEXA* variants such as the relatively common pseudodeficiency (PD) allele (present in 35% of non-Jewish enzymatically defined carriers). Hex A in PD individuals has a reduced ability to hydrolyze artificial substrates but has normal activity toward G_{M2} ganglioside. Heterozygotes for the deficiency allele are scored as carriers for TSD on the serum enzyme test, however compound heterozygotes for one of these alleles and a disease-causing mutation are clinically normal (Thomas et al, 1982; Grebner et al, 1986; Triggs-Raine et al, 1992). DNA diagnostic procedures for PD alleles are available (Triggs-Raine et al, 1992).

Individuals carrying the B1-TSD allele are misclassified since screening programs use 4MUG as a substrate. Enzyme test results usually fall in the low non-carrier range or in the 'gray' zone area (Conzelmann et al, 1985). The frequency of this type of mutation, however, is quite low except among Portuguese TSD patients (Dos Santos et al, 1991).

Advances in molecular biology have provided the means to identify and characterize TSD mutations. The use of DNA testing as a replacement for serum hexosaminidase assays is feasible only if a very high percentage of heterozygotes in the target population carries one of a small number of defined mutant alleles.

In the Ashkenazi Jewish population three *HEXA* mutations (+TATC$_{1278\text{-}1281}$, IVS-12, +1 G->C and G269S) account for over 90 % of mutant alleles, rendering DNA diagnostics a feasible approach to carrier screening in this community. In attempting to compare the value of the two types of tests it should be borne in mind that neither the enzyme or DNA test has a sensitivity of 1, however each test is likely to misclassify a different type of person. It has, therefore been proposed that the enzyme assay should remain the primary TSD screening test and should be complemented by DNA diagnostics (Triggs-Raine et al, 1990; Paw et al, 1990a; Grebner and Tomczak, 1991; Landels et al, 1991; Fernandes et al, 1992a). For detection of TSD genes among families not belonging to the major at-risk populations, the serum enzyme test will certainly play a dominant role since screening for more than 50 different known 'private alleles' is too time consuming. However, for this

group, enzymatically defined carriers should also be screened for the presence of PD alleles.

The establishment of these TSD screening programs has led to attempts to define criteria to be applied for the initiation of other programs focused on the prevention of a specific genetic disorder. These criteria include, i) a high incidence of the disorder in the target population, ii) a cost-effective, relatively simple screening test with a high specificity and sensitivity and iii) the availability of follow-up services for individuals with a positive result such as genetic counselling and prenatal diagnosis (Scriver and Hechtman, 1979). TSD fulfills all these criteria and has therefore become the prototype for the screening of other genetic recessive disorders. The TSD carrier frequency in the Ashkenazi Jewish population is 1 in 31 (Kaback et al, 1993), the screening test is a relatively simple enzyme assay with high specificity and sensitivity for distinguishing phenotypes and prenatal tests safely and accurately identify those fetuses affected with the disorder (Kaback et al, 1977).

Probably the most important initial factors ensuring the success of TSD screening was educating the Ashkenazi Jewish population about TSD and promoting the participation of community leaders as well as volunteers. A continual assessment of the effects of the screening program on the population and the periodic quality control tests monitoring the accuracy of screening by use of coded serum and leukocyte samples, are also key to the success of TSD screening. The psychosocial components evaluated included educational

effectiveness, factors determining compliance and non-compliance and possible individual, family or community stigmatization effects.

As of June 1992, more than 36 000 heterozygotes have been screened, 1 056 couples at risk for having a TSD offspring have been identified and a total 2 416 high-risk pregnancies have been monitored. Sixty-two thousand individuals are screened for TSD annually, worldwide. The reduction in the frequency of infants born with TSD, in the Ashkenazi population, is greater than 90 %. In contrast, in non-Jewish populations, who have not been the main target of screening programs, the incidence of the disease has remained the same, demonstrating the effectiveness of the screening effort (Kaback et al, 1993).

The prenatal diagnosis component of TSD screening has traditionally relied on amniocentesis at 14 weeks of gestation with enzyme assays or DNA diagnostics performed on cultured amniocytes 2-3 weeks later. More recently chorionic villus biopsy sampling appears to offer the possibility of earlier (8-10 week gestation) results.

1.10 Structure-function studies at the HEXA gene

1.10.1 Expression systems

The clinical, biochemical and genetic aspects of TSD have been extensively studied. However, structural analysis of wild-type and mutant subunits has been hampered by a

lack of x-ray crystallographic data. Currently, structure-function studies are limited to biochemical analysis of purified normal enzymes and site-directed mutagenesis experiments in *in vitro* systems. The latter approach involves the expression of mutant α and/or β cDNA's with codon substitutions for residues that have been identified as functionally important either because of evolutionary conservation or naturally occurring mutations at these sites that produce gene products with altered functions. As a result of analysis of properties of mutant forms of hexosaminidases a number of residues have been implicated in either catalytic function or protein folding.

Several expression systems for the study of *HEXA* and *HEXB* mutant alleles have been reported. These include i) SV40-transformed, human, Sandhoff disease (SD) fibroblast and Epstein-Barr virus transformed SD lymphoid cell lines (Momoi et al, 1985; Maret et al, 1985), ii) an Epstein-Barr virus transformed, human, TSD lymphoid cell line (Maret et al, 1985), iii) the yeast, Saccharomyces cerevisiae (Prezant, 1990), iv) Sf9 (insect) cells (Boose et al, 1990) and v) COS (SV40-transformed monkey, kidney cells) (Nakano et al, 1990).

Following SV40-transformation of SD fibroblasts the level of hexosaminidases remained unchanged. Similarly, the SD lymphoid line shows a severe deficiency of Hex A and B and the TSD line has an abnormal isoenzyme composition. In neither of these three human cell lines or in yeast have studies on mutant *HEXA* or *B* alleles been performed.

There has been one report, however, of the transfection of a mutant *HEXA* cDNA into TSD fibroblasts (Cotton, 1993).

Human *HEXB*, when expressed in Sf9 cells using the baculovirus as vector, produces an active Hex B of similar size to that of the enzyme produced in human cells. However, Sf9 cells and fibroblasts perform different post-translational processing reactions. Consequently, human Hex B from Sf9 cells contains oligossaccharide structures which are different from those found in fibroblast Hex B and the former enzyme does not contain the mannose-6-phosphate recognition marker necessary for targeting. Nevertheless, human enzyme expressed in Sf9 cells is catalytically active and is a substrate for phosphotransferase indicating that correct protein folding has occurred.

The baculovirus expression system also offers the possibility of an efficient high-yield system for hexosaminidase purification. Under optimal conditions the yield of secreted, active human Hex B is 1mg/l. Subsequent isolation and purification of enzyme from the medium is relatively simple using standard, established technology.

The COS cell is the only system that has been used for expression of mutant *HEXA* and *HEXB* alleles. Expression analysis, in this system, however, is rather complex due to the presence of endogenous simian hexosaminidases and to the heterodimeric structure of human Hex A. Furthermore, when cells are transfected with mutant *HEXA* cDNA's, the

low levels of 4MUGS hydrolysis (relative to mock transfected cells) is difficult to measure making biochemical distinction between mutations difficult to achieve.

Three strategies for enriching, or isolating hexosaminidase activity in COS transfectants due to expressed human enzymes have been compared by Brown and Mahuran (1993). The first and most commonly used strategy is transfection with a vector carrying only the mutant *HEXA* cDNA of interest. Although the level of 4MUGS hydrolysis (relative to mock transfectants) in the wild-type transfectants is 3 to 5 fold, *HEXA* mutant cDNAs analysed in this manner do not yield significant levels of residual 4MUGS hydrolysis. This approach has been often used to confirm that a mutation confers some form of G_{M2} gangliosidosis but it can not generally permit distinction between alleles conferring different clinical phenotypes.

The second approach takes advantage of the evolutionary relationship between the *HEXA* and *B* genes. The two subunits, which have 57 % identity, presumably contain conserved domains with similar functions. *HEXA* mutations were introduced into the corresponding codon of the *HEXB* gene and studied in the isoenzyme Hex B. This approach proved to be valid for the putative active site mutation (αR178H) indicating that the arginine residue at β–R211 (Brown and Mahuran, 1991) plays a similiar role in both isozymes. This strategy, as well as the following approach outlined below, depends upon separation of the expressed enzyme from the endogenous species. In practice, separation is achieved

by immunoprecipitation of the human enzyme using an antiserum that recognizes only the human protein.

The third strategy is cotransfection with mutant α and normal β cDNA to form a species of human Hex A with a mutation only in the α–subunit. Human Hex A activity is determined following immunoprecipitation with an antisera specific for human β-chains. In this system 4MUGS hydrolysis by human Hex A carrying one of the three mutations G269S, G250D and E482K, was higher than that in patient fibroblasts, presumably due to overexpression of human *HEXA* and *HEXB* cDNA's in transfected cells. The residual activities were 46%, 26% and 19%, respectively, relative to wild-type *HEXA/HEXB* transfectants. These mutants produce 4-6%, 2-3% and < 1% of wild type levels of 4MUGS hydrolysis in patient fibroblasts. This expression system, however, is more sensitive than the *HEXA* expression systems described above since residual enzyme levels were inversely correlated with the severity of the clinical phenotype associated with each of the mutations.

1.10.2 Putative active site residues at the *HEXA* locus

All glycosyl hydrolases are believed to employ a general acid catalysis mechanism requiring two critical residues; i) a proton donor and ii) a nucleophile/base (Sinnott, 1990). Hydrolysis can either result in the retention or inversion of the anomeric configuration. The retaining mechanism involves the protonation of the glycosidic oxygen by the acid catalyst and a subsequent nucleophilic attack by the base to release the

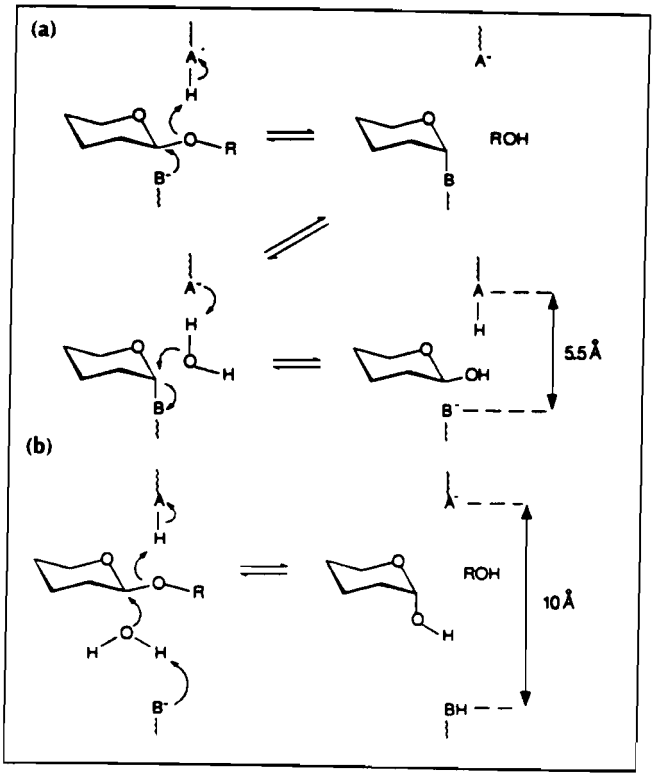

Figure 4: The two major mechanisms of glycosidic bond hydrolysis. See text for details. (From Davies and Henrissat, 1995)

aglycone (Fig 4). In the inverting mechanism, the protonation of the glycosidic bond and the departure of the aglycone are accompanied by a concomitant attack of a water molecule which is activated by the base. Although there is no direct evidence bearing on the mechanism of hexosaminidase-catalyzed hydrolysis, acid-base catalysis in these enzymes is likely to result in retention of configuration since chitinases with primary sequences homologous to hexosaminidase, such as the *Serratia marcescens* chitobiase (CHS), have been shown to work by a retention mechanism (Tews et al, 1995).

Aspartic acid and/or glutamic acid residues have been identified as components of the active sites of many glycosyl hydrolases, however other residues may participate in catalysis. Tyrosine has been shown to stabilize the transition state in viral neuraminidase- and bacterial sialidase-catalyzed reactions (Burmeister et al, 1993; Crennel et al, 1993). Piszkiewicz and Bruice (1968) suggested that the C2 acetamido group of the substrate can participate in β-glycoside hydrolysis. Hydrolysis of N-acetylglucosamine-containing saccharides by egg white lysozyme occurs faster than that of C2-hydroxyl-substituted sugars with overall retention of anomeric configuration suggesting that an 'on enzyme' catalytic base may not be required (reviewed in Gideon and Henrissat, 1995).

Several strategies may offer insight into the location of putative active site residues. These include i) naturally occuring mutations which specifically affect catalysis, ii) conservation of amino acid sequence, iii) three dimensional structural data (eg: x-ray

crystallography) and iv) protein labeling with 'suicide substrates' or group specific reagents.

1.10.2.a. Naturally occuring mutations

At the *HEXA* locus, the naturally occurring mutations known as B1 mutations are thought to affect residues having catalytic function. Mutations in three codons in the *HEXA* gene are associated with the B1 biochemical phenotype: R178 (Ohno and Suzuki, 1988), V192 (Ainsworth and Coulter-Mackie, 1992)and D258 (Fernandes et al, 1992b). Amino acid 178 has been extensively studied and the role of D258 is in part the subject of this thesis.

Brown and Mahuran (1991) investigated the role of R178 by expressing the homologous and conservative β-subunit mutation R211K in COS cells. The mutant Hex B exhibited a drastically reduced level of catalytic activity; a 400-fold decrease in Vmax relative to normal enzyme and no change in Km. The in vivo stability of Hex B-R211K and its rate of processing were also unaffected suggesting that the loss of catalytic function is unlikely to be merely a secondary consequence of misfolding.

Inactivation of normal Hex B by dicarbonyl reagents which specifically react with 'active' arginines, support the hypothesis that αR178/βR211 is involved in catalysis. In contrast, both dicarbonyl reagents 2,3 butanedione and phenylglyoxal caused no loss of enzyme activity in the Hex B-R211K which further supports this hypothesis.

1.10.2.b. Homology and structure analysis

The identification of potential active-site residues, on the basis of invariant amino acid residues, requires a detailed primary structure analysis of evolutionarily related glycosyl hydrolases. In 1991, Bernard Henrissat proposed an alternative classification system to the IUB Enzyme Nomenclature for glycosyl hydrolases which was based on amino acid sequence similarity rather than substrate specificity or reaction mechanism. Since primary sequence directs protein folding, the new classification system may achieve a more accurate grouping of enzymes based on similiarity of higher order structures (Gideon and Henrissat, 1995).

Glycosyl hydrolases have been classified into at least 35 different families. A family is defined as having at least two sequences that display significant amino acid similarity and no similarity toward members of other families. The Swiss-Prot database entries now contain in their comment section (CC) the family to which the glycosyl hydrolase belongs. Hexosaminidases belong to family 20 which has at least 5 members and 5 other potential members. In contrast, under the EC system, which classifies enzymes according to the type of reaction they catalyze and on their substrate specificity, human hexosaminidase is classified with approximately 80 other enzymes. A comparison between the EC and the Henrissat classification systems indicates that (i) individual members of the same family may have different EC entries and (ii) enzymes that belong to non-related families may have similar substrate specificity. The older EC cannot take

into account evolutionary events and is not appropriate for enzymes showing broad specificity.

Since the publication of the Henrissat classification system a tremendous amount of information has been compiled furthering the understanding of glycosyl hydrolases. Today, it is known that catalytic residues are absolutely conserved within a family. This information permits investigators to deduce active residues of unknown enzymes by comparison to corresponding residues in a family member whose active site have been identified. Baird (1990) and Py (1991) used this kind of homology analysis to identify putative invariant active site residues whose function they later confirmed experimentally. Furthermore, members of each family generally share the same folding characteristics enabling homology modelling assuming that the 3D structure of one of the members is known (Henrissat, 1991; Henrissat and Bairoch 1993).

The first crystallization and three dimensional structure analysis of a member of family 20 was reported by Tews and colleagues (1995) for the enzyme chitobiase (CHS) from *Serratia marcescens*. CHS is 858 amino acids long and folds into four domains. The catalytic domain has an eight stranded αβ-barrel fold. As is typical of αβ–barrel enzymes (Farber and Petsko, 1990), the active site of CHS is at the C-terminal end of the barrel. The catalytic residues (E540 and E739) are located in loops 4 and 8. The combination of homology analysis, using the Henrissat classification system and comparison with CHS tertiary structure data enabled the identification of three strong

active residue candidates in the α-subunit of human hexosaminidase (E307, E323 and E462). The expression analysis of the Hex A homologues of these active site residues is part of this thesis.

1.10.2.c. Protein labelling

Liessem et al (1995), using the photoaffinity label, [^{3}H]-1-ATB-GalNAc, provided evidence for the involvement of E355 in the β-subunit as a component of the active site of HexB. This residue is the β-subunit homologue of E323 in the α-subunit which is tested as an active site residue in these studies.

1.11 Objectives:

Historically, the present study began after the cloning of the *HEXA* gene when only three *HEXA* mutations had been identified. The three mutations identified were known to account for over 90% of mutant TSD alleles in the Ashkenazi Jewish population. The screening test used to identify carriers is an enzyme-based test. The first objective was to compare DNA diagnostics to the enzyme test in an attempt to improve classification of carrier individuals.

The second objective was to identify and characterize novel TSD mutations since it became apparent that TSD exhibited allelic heterogeneity. Four novel mutations were identified. One of these, which affects the splicing of *HEXA* mRNA primary transcript, was further analyzed using competitive PCR. The identification of a putative active site

mutation among the four novel alleles characterized led to the third objective which was to establish an improved expression system for the study of TSD mutant alleles that would permit analysis of low levels of Hex A activity. The commonly used expression system, the COS cell system, has endogenous (simian) hexosaminidase making the analysis of *HEXA* mutants rather challenging. A novel expression system was established in a SV40 transformed, neuroglial (NG) cell line obtained from a TSD patient. These cells synthesize no α-subunit and have endogenous hexosaminidase A levels approximately 1% of those of wild-type neuroglial cells.

With the characterization of an improved system for the expression of *HEXA* mutations a fourth objective emerged which was the identification of active site residues of the α-subunit of Hex A. Four active site candidate residues were tested by expression of Hex A mutant alleles carrying conservative substitutions at the appropriate codons. These residues were proposed as candidates based on the strategies described above.

CHAPTER 2

SPECIFICITY AND SENSITIVITY OF HEXOSAMINIDASE ASSAYS AND DNA ANALYSIS FOR THE DETECTION OF TAY-SACHS DISEASE GENE CARRIERS AMONG ASHKENAZIC JEWS

Specificity and sensitivity of hexosaminidase assays and DNA analysis for the detection of Tay-Sachs disease gene carriers among Ashkenazic Jews

Running title: Enzyme vs DNA tests in Tay-Sachs Carrier Screening

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2.1 ABSTRACT

Tay-Sachs disease (TSD), a neurodegenerative disorder resulting from a deficiency of the lysosomal enzyme hexosaminidase A (HexA), clusters in Ashkenazic Jews. Population-based screening programs to detect carriers of TSD genes by means of HexA assays have been active since the 1970's. The recent characterization of 3 mutations in the HEXA gene (in exon 7, exon 11 and intron 12), which account for over 90% of HEXA mutations in Ashkenazim, appeared to offer better options for screening and diagnosis. The relative frequencies of the three mutations in Montreal are similar to those reported in four other North American populations. We compared enzyme and DNA analyses to determine specificity and sensitivity of each test when the other was used as the confirmatory procedure. Neither procedure has a sensitivity of 1.0. Maximum sensitivity and specificity were achieved by using both tests together. The findings here are likely to apply to most cases where the variant screened enzyme phenotype can result from more than one mutation.

Key words: genetic screening, HEXA mutations, population genetics

2.2 INTRODUCTION

Tay-Sachs disease (TSD) is a recessively inherited, neurodegenerative disorder resulting from deficient activity of the lysosomal enzyme ß-N-acetylhexosaminidase A (Hex A), which leads to the accumulation of GM_2 ganglioside in lysosomes [Sandhoff et al., 1989]. Hex A is a heterodimer with α and ß subunits (Hex $\alpha\beta_a\beta_b$) encoded by genes on chromosome 15q23-q24 and 5q13 respectively; [Takeda et al, 1990; Sandhoff et al., 1989]. Mutations in the former gene (symbol, HEXA) cause TSD. HEXA spans 35 kb and has 14 exons [Proia and Soravia, 1987]. At least 40 mutations have been characterized. The three most common occur in the Ashkenazic Jewish population, a high risk group. They are: (1) a 4 bp insertion (TATC) in exon 11 [Myerowitz and Costigan, 1988], (2) a splice junction mutation (+1, G->C transversion) in intron 12 [Arpaia et al., 1988; Myerowitz et al., 1988; Ohno and Suzuki, 1988] and (3) a G805A transition in exon 7 [Navon and Proia, 1989]. The first two are associated with infantile TSD; the third with an adult-age onset variant of GM_2 gangliosidosis. TSD, which also occurs at high frequency in certain French Canadian demes [Andermann et al., 1977], is mainly the result of a 7.6kb deletion, removing exon 1 and part of intron 1 [Myerowitz and Hogikyan, 1986, 1987].

The ability to distinguish between non-carriers and carriers of HEXA mutations, by relatively simple enzyme (phenotype) assays and classification methods, led to the establishment of population-based screening programs in high-risk populations [Kaback

et al., 1977a]. The screening tests use an enzyme assay to differentiate between the genetically related isoenzymes Hex A and Hex B [Delvin et al., 1974; Kaback 1973; Lowden et al., 1973; O'Brien et al., 1969]. Gold et al (1974) showed that one could discriminate carriers from non-carriers best by taking the non-independent Hex A and Hex B values together, than by using either enzyme value alone.

The phenotype test (enzyme assay) has its limitations [Gold et al., 1974; Isakssim et al., 1985; Kaback et al., 1977; Kolodny, 1977; O'Brien et al., 1970]. Accordingly, the possibility of screening for carriers by DNA analysis has its attractions. Comparison of results obtained by DNA analysis and by enzyme test has been a subject of recent interest [Grebner and Tomczack, 1991; Paw et al., 1990; Triggs-Raine et al., 1990]. Here we compare DNA and enzyme tests in the Ashkenazic Jewish population of Montreal. We report the relative frequency of the exon 7, exon 11 and intron 12 mutations in this population; compare the results of our DNA and enzyme tests, and compare our findings with previous studies. A preliminary report of our own study has appeared [Fernandes, M. et al., 1990]. The relevance of such studies has long been recognized in that ..."collaborative study groups should be continuously assessing the information derived from screening, evaluating new developments, and reviewing social and biological consequences of their work" [WHO, 1968].

2.3 MATERIALS AND METHODS

2.3.1 Sample selection. Subjects (n = 78) for this study were ascertained through the Quebec TSD carrier screening program [Beck et al., 1974; Clow and Scriver, 1977]. Our laboratory participates in the quality control program [Kaback et al, 1977b) offered by the International Tay-Sachs Disease Testing Quality Control and Data Collection Center and has achieved perfect scores.

Files for the past decade were reviewed. Individuals classified as carriers by the screening test, who also had a close relative tested and classified as a carrier in our program, were selected on the assumption that they had a high probability of being true carriers of HEXA mutations. Normal subjects were recruited from the same program. Venous blood samples were used to prepare serum aliquots (for phenotype assay) and leukocyte DNA (for mutation analysis).

2.3.2 Classification by phenotype assay. We measured enzyme (Hex A) activity by a conventional semi-automated method [Delvin et al., 1974]. Classification of the metrical value was done according to the statistical method of Gold et al (1974), based on quadratic discriminants. Individuals with putative false test results were restudied wherever possible by repeating the enzyme assay on fresh samples (serum and leukocytes) using 4MUG and 4MUGS substrates [Bayleran et al., 1984].

2.3.3 Classification by DNA analysis. DNA was isolated and analyzed for the exon 7 (Navon and Proia, 1989], exon 11 [Myerowitz and Costigan, 1988; Triggs-Raine, 1990] and intron 12 [Myerowitz, 1988] mutations as described.

2.3.4 Additional mutation analysis. DNA samples from putative carriers (deficient serum Hex A activity, negative by DNA analysis for the aforementioned mutations) were further analyzed by strand separation gel electrophoresis [Orita et al., 1989] under modified conditions for HEXA [Triggs-Raine et al, 1991].

2.4 RESULTS

The sample population comprised 78 individuals. They were classified as 28 carriers and 49 non-carriers and one doubtful as to genotype by the enzyme assay (Table I). Among the carriers classified by their HexA value, there were 22 with the exon 11 mutation, 4 with the intron 12 mutation, and 1 with the exon 7 mutation; one person confidently identified as a carrier by Hex A assay had none of these mutations. Three subjects classified initially as non-carriers by enzyme assay were each identified as carriers of the exon 11 mutation by DNA analysis. Accordingly, they had putative false negative enzyme screening test results. One of these individuals had Hex A and B values that did not allow classification either as a carrier or a non-carrier (classified as "doubtful" in Table I). The other two persons had enzyme values unambiguously distributed in the non-carrier region on repeated tests. Mother, brother and daughter of one of these persons each carries the exon 11 mutation and they are all unambiguous carriers by enzyme values. These findings imply that unknown factors are responsible for the discrepancy between the enzyme and DNA test results and that some individuals bearing the exon 11 mutation can have normal enzyme values.

The relative frequencies of the HEXA mutations in the Montreal population are: 0.81 for the exon 11 mutation, 0.13 for the intron 12 mutation, 0.03 for the exon 7 mutation and 0.03 for unidentified mutations. Relative frequencies of the three major mutations are

similar in the five different North American Ashkenazi populations represented in these studies (Montreal, Toronto, Boston, Philadelphia, and Los Angeles)(Table I).

We estimated so-called "sensitivity" and "specificity" of our enzyme (screening) test by comparing it with the results obtained by DNA analysis on the same samples (Set A, Table II). Specificity (the probability that non-carriers will be excluded by the enzyme test) was 0.98. Sensitivity (the probability that a carrier will be identified by the enzyme test) was 0.90 in our population sample; note that 2 of the 3 "non-carrier" persons bearing TSD alleles had unambiguously normal Hex A and Hex B values. Specificity and sensitivity were 0.94 and 0.96 respectively when DNA analysis is the "screening" test and the enzyme assay is used as the reference test (Set B, Table II).

2.5 DISCUSSION

We report relative frequencies of the three major HEXA mutations (exon 7 substitution; exon 11 insertion; intron 12 splice-junction substitution) associated with deficient Hex A activity in several Ashkenazic Jewish populations in North America. Findings in the Montreal population are similar to those found in Toronto, Boston, Philadelphia and Los Angeles. In the United Kingdom [Landels et al., 1991], relative frequencies of the exon 11 and intron 12 alleles are not dissimilar from those in North America. Together, the three mutations appear to account for about 90 percent of HEXA mutations in Ashkenazic Jews; relative frequencies vary modestly between geographic regions.

We compared classification of persons by enzyme (Hex A) assay and by mutation (HEXA) analysis. We had predicted earlier [Gold et al., 1974] that about 2% of "obligate" TSD carriers would have false negative tests by enzyme assay and about 6% of non-carriers would be false positive. Two persons classified unambiguously but wrongly as non-carriers by enzyme assay in the present study had HEXA mutations. Another person who had an ambiguous "non-carrier" test result also harboured an exon 11 mutation. The modifiers of phenotype acting in these subjects are not known. The goal of the screening test is to maximize sensitivity with least cost to specificity [Gold et al, 1974], nonetheless a small percent of screened persons classified as "non-carriers" for a Tay-Sachs mutation by a reliable enzyme assay (this study and Triggs-Raine et al, 1990; see Table I) actually

had one of the prevalent mutations. The finding corroborates our earlier prediction; it has not been commented on before now.

One individual in the Montreal study classified as a carrier by phenotype assay, was negative by DNA analysis for the three most common mutations. SSCP (single strand conformation polymorphism) analysis did not reveal other known or unknown mutations. Whereas this individual could be classified as a putative false positive on the basis of enzyme and DNA test results, we recognize that not all mutations will be detected by SSCP analysis and that our mutation analysis is not yet complete.

Whereas the findings reviewed here show that the serum enzyme assay by itself is the most efficient test for population screening, they also indicate that samples with enzyme (Hex A) values which are indeterminate and elude secure classification should be tested at the DNA level as well. This refinement will improve classification of enzyme based screening tests for carriers of HEXA mutations and it will have relevance for families seeking prenatal diagnosis.

The findings in the present study and in three others [Grebner et al., 1990; Paw et al., 1990; Triggs-Raine et al., 1990] are compared in Table I. We draw attention to a significant difference in study designs. Only the serum enzyme assay (the conventional screening test) was performed in our study. In the others, both serum and leukocyte assays were used for classification; a combined test is used for diagnosis, but not

uniformly for screening. Accordingly, we used only our data to evaluate relative specificity and sensitivity of enzyme and DNA methods. We show that sensitivity is not 1.0 for either the enzyme test or for DNA analysis (Table II). The limitations of the enzyme test are well-known; we see here that DNA analysis will also have limitations until all mutations can be detected routinely. But when both methods are used iteratively, the predictive value of either test alone is improved. TSD carrier screening programs will probably use an efficient combination of both methods (enzyme and DNA) as the best strategy for now.

The present and allied studies of Tay-Sachs carrier screening are surely prototypes for all cases of carrier screening by phenotype tests.

TABLE I. Classification of Persons by Combined Phenotype (Enzyme) and DNA (Mutation) Analysis

	A		B			
			Mutation in Set A identified by DNA Analysis			
Source	Classification		Exon 11	Intron 12	Exon 7	Unidentified[a]
This study[b]	Non-carrier	49	2			
	Doubtful	1	1			
	Carrier	28	22	4	1	1
Triggs-Raine et al [1990][c]	Non-carrier	152	1			
	Carrier	216	140	32	5	39
Paw et al [1990][c]	Carrier	139	114	14	4	7
Grebner & Tomczak [1991][c]	Carrier	122	89	21	5	7
	Relative Frequency:		0.725	0.140	0.029	0.106

a) Unambiguous phenotype classification but none of the 3 mutations listed in Set B were found by DNA analysis.

b) Classified by serum assay alone (conventional enzyme screening test).

c) Classified by combined serum assay and leukocyte assay (modified "screening" test).

TABLE II. Estimates of Specificity and Sensitivity of Tests for Detection of TSD Carriers*

		"Screening" Test				
		Set A by enzyme (Hex A) assay			Set B by DNA (mutation) analysis[a]	
		Negative	Doubtful	Positive	Negative	Positive
"Confirmatory" test[b]	Negative (specificity)	47 (0.98)		1	47 (0.94)	2
	Doubtful				1	
	Positive (Sensitivity)	2	1	27 (0.90)	1	27 (0.96)

*) Screened samples (n = 78) from Montreal population only (present study) used for analysis.

a) DNA samples were analyzed for the exon 7, exon 11 and intron 12 mutations.

b) The "confirmatory" test is mutation analysis when the screening test is the enzyme assay (Set A) and the enzyme assay when the "screening" test is mutation analysis (Set B)

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Connecting text - Chapter 2 to Chapter 3 and 4:

With the advent of molecular biology and the possibility of screening for TSD at the DNA level, the complete characterization of the underlying allelic heterogeneity at the *HEXA* locus became a priority.

Four novel TSD mutations were identified. Three were identified in two B1 patients, reported in chapter 3, and the fourth, identified in a chronic TSD patient, is reported in chapter 4.

CHAPTER 3

A NEW TAY-SACHS DISEASE B1 ALLELE IN EXON 7 IN TWO COMPOUND HETEROZYGOTES EACH WITH A SECOND NOVEL MUTATION

A New Tay-Sachs Disease B1 Allele in Exon 7 in two Compound Heterozygotes each with a second Novel Mutation

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3.1 ABSTRACT

Three novel Tay-Sachs Disease (TSD) mutations have been identified in two unrelated, non-Jewish compound heterozygous patients. A G772C transversion mutation causing an Asp258His substitution is shared by both patients. The mutant enzyme had been characterized, on the basis of previous kinetic studies (1) as a B1, or α-subunit active site mutation. This is the first B1 mutation not found in codon 178 (exon 5). A C508T transition causing an Arg170Trp substitution also occurred in one of the patients. The third mutation is a two base deletion occurring in exon 8 involving the loss of either nts 927-928 or 929-930 in codon 310. The deletion creates an inframe termination codon 35 bases downstream. The Arg170Trp mutation was also detected in a third unrelated TSD patient. In both families this allele was traced to French Canadian ancestors originating in the Estrie region of the province of Quebec. This mutation is the third TSD allele unique to the French Canadian population and the ancestral origins of the carrier parents are distant from the center of diffusion of the more common 7.6kb deletion mutation which is in the eastern part of the province.

3.2 INTRODUCTION

Tay-Sachs disease (TSD) is an autosomal recessively inherited lysosomal storage disorder characterized by the accumulation of GM_2 ganglioside in the neurons of the central cortex. The deficient enzyme, hexosaminidase A (Hex A), is a heterodimer of α and ß subunits encoded by the HEXA and HEXB genes respectively. Mutations in the HEXA gene cause TSD, which, in its classical form produces blindness, paralysis and dementia in infants. The frequency of TSD is elevated in the Ashkenazi-Jewish and French-Canadian populations (2).

Over 40 different mutations in the HEXA gene have been identified. Their enumeration is useful in understanding the broad clinical spectrum of the GM_2 gangliosidoses, in investigating structure-function relationships in the HEXA gene product and in studying the genetic structure of populations.

B1 mutations occur in the HEXA gene resulting in loss of α-subunit catalytic function and reduction of hydrolysis of GM_2 ganglioside and the sulfated synthetic substrate 4-methylumbelliferyl-ß-D-N-acetylglucosamine-6-sulfate (4MUGS). These mutations are associated with a form of enzyme which has electrophoretic and thermolability properties characteristic of Hex A but catalytic activity only toward electrically neutral substrates such as 4-methylumbelliferyl-ß-D-N-acetylglucosamine (4MUG).

We studied two patients with infantile TSD whose serum and fibroblasts contained heterozygote levels of Hex A. Biochemical studies on patients 1 and 2 were described previously (1). Both patients appeared to be heterozygous for the B1 phenotype having virtually no capacity for hydrolysis of the sulfated hexosaminidase A substrate 4MUGS.

3.3 METHODS

3.3.1 Single-stranded conformational polymorphism (SSCP) Analysis was performed according to Orita et al (3) as modified by Triggs-Raine et al (4). PCR amplification of HEXA exons and flanking sequences was according to Triggs-Raine et al (4).

3.3.2 Sequencing Direct sequencing of amplified PCR products was performed by the method of Sanger et al. (5).

3.3.3 Other Details of procedures used for confirmation of mutations are reported in the legend to figure 2. Nucleotide position numbers refer to cDNA sequence and commence with the ATG initiation codon.

3.4 RESULTS

In all cases, exons carrying the mutations were detected using SSCP analysis of genomic DNA (data not shown). Sequence alterations in amplification products are shown in figure 1. A G772C transversion in exon 7 results in an Asp258His substitution in patients 1 (top) and 2 (data not shown). In patient 1 and his mother a C508T transition in exon 5 results in a Arg170Trp substitution (middle). In patient 2 an exon 8 deletion of a CT dinucleotide at positions 927-928 or 929-930 occurs in codon 310 producing a "double ladder" in sequencing gels (bottom). An inframe stop codon TGA 35 bp downstream (bottom) is created with the result that this mutation is expected to produce no immunoreactive protein due to premature termination of translation.

The mutations in exons 5 and 8 could be confirmed by restriction enzyme digestion of amplfiied genomic DNA sequences of the proband and parents. The mutation in exon 5 abolishes a ScrF1 site. Thus the presence of a 165 bp undigested fragment in both patient 1 and his mother and its absence in the patient's father and in control DNA confirmed the findings of the sequencing gel and identifies the mother as the source of the exon 5 mutation (figure 2A). The exon 8 mutation introduces a Pvu II site. The presence of restriction fragments of 162 bp and 105 bp in DNA amplified from both patient 2 and his father following Pvu II digestion confirms this mutation and identifies the father as the origin of this mutant allele (figure 2A).

The mutation in exon 7 was confirmed by allele-specific oligonucleotide hybridization to amplified exon 7 sequences. This mutation was detected in both patients 1 and 2 (figure 2B) and was of paternal and maternal origin respectively. Sequencing of exon 5 in both patients ruled out the presence of any of the previously described codon 178 B1 alleles (figure 1, data shown for patient 1 only).

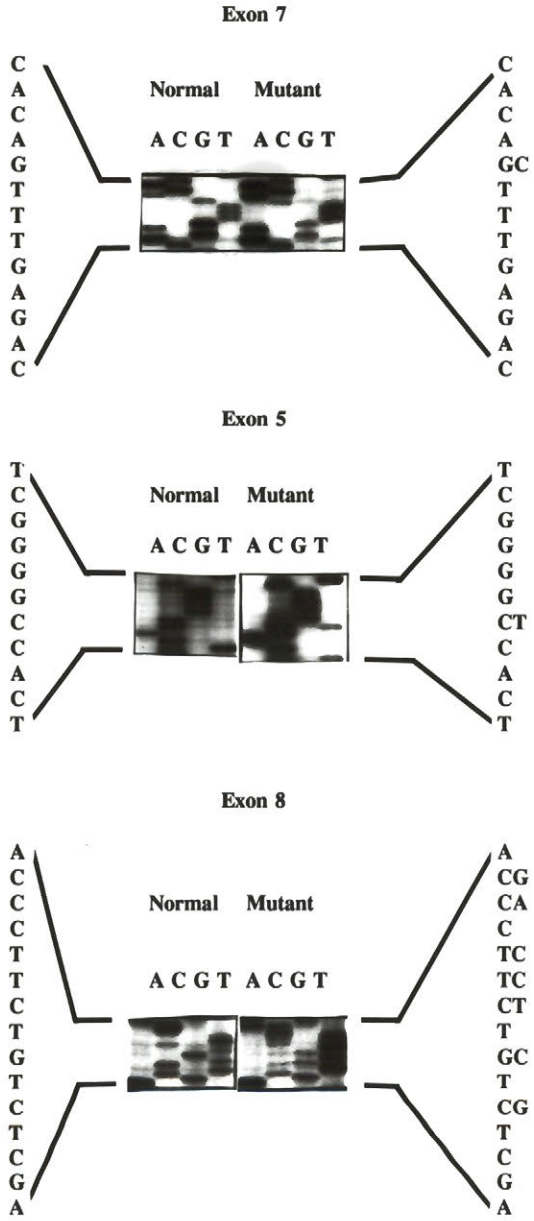

Figure 1 Sequences of normal and mutant alleles in exons 7,5 and 8. Sequencing reactions were performed directly on PCR amplification products. Top: patient 1, Middle patient 1, Bottom: patient 2. A double sequencing ladder occurs in the bottom gel beginning at the fifth base from the bottom. At positions where only one base occurs within this double ladder the effect of the dinucleotide deletion is to bring identical bases into correspondance.

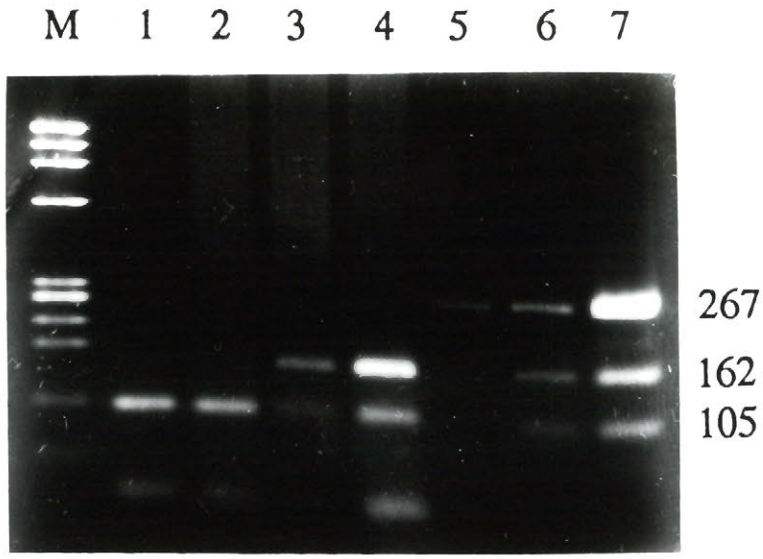

Figure 2A Detection of mutations in genomic DNA in family members by restriction enzyme digestion. Lane M, Molecular Weight Markers (HaeIII-digested ϕX174 DNA); Lanes 1-4 ScrF1 digests. Lane 1, normal control; Lane 2, patient 1's father; Lane 3, patient 1's mother; Lane 4, patient 1. Lanes 5-7, PvuII digestion. Lane 5, normal control; Lane 6, patient 2; Lane 7, patient 2's father.

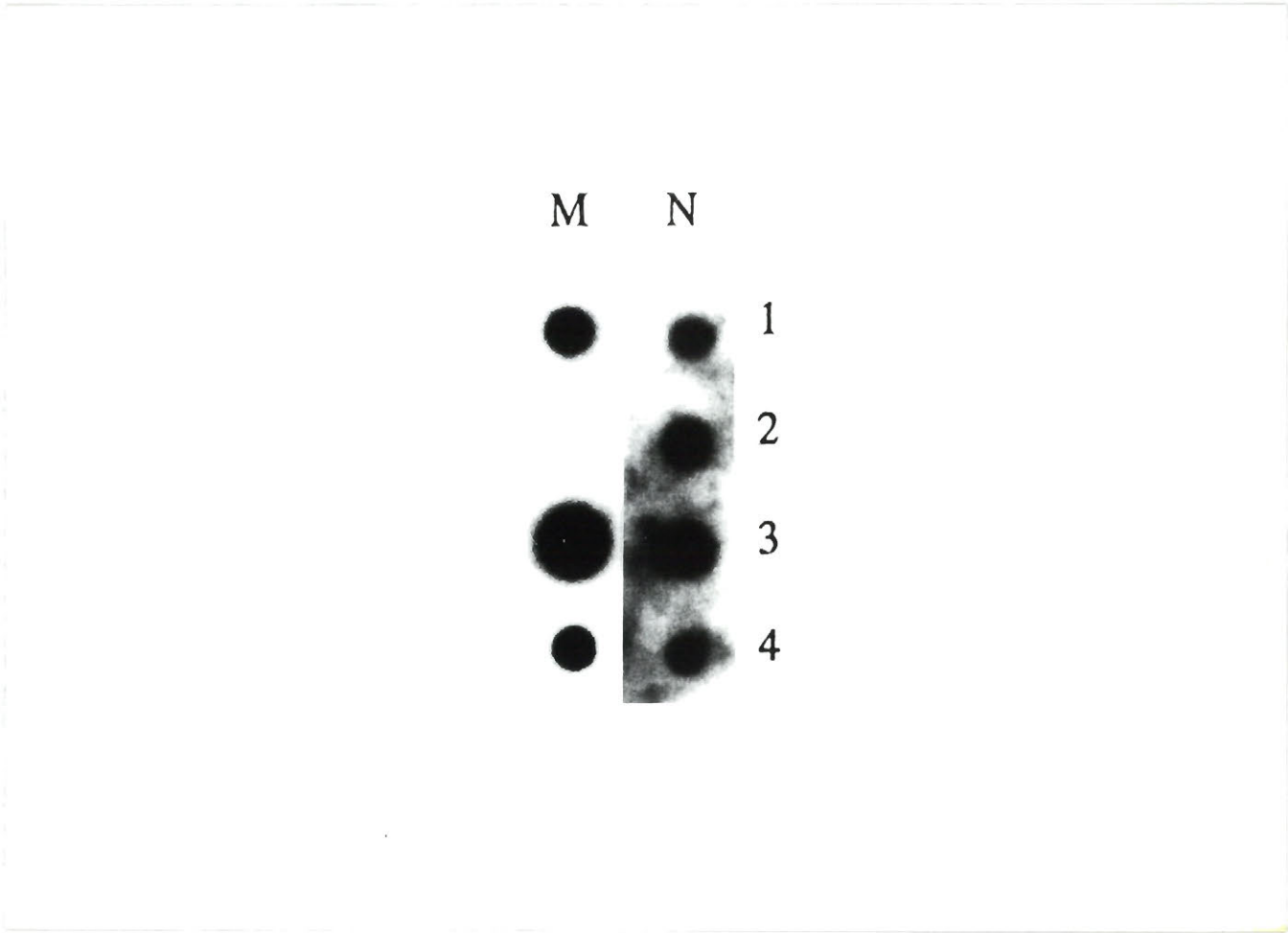

Figure 2B Detection of mutations in genomic DNA in family members by allele-specific oligonucleotide hybridization. Normal allele-specific oligonucleotide probe: GAGTTTGACACTCCT GGCCAC, mutant allele-specific oligonucleotide probe: GAGTTTCACACTCCTGG CCAC. Amplified DNA loaded on row 1, patient 1; row 2, normal control; row 3, patient 2; row 4, patient 2's mother.

3.5 DISCUSSION

The three novel mutations extend our knowledge of the range of mutant genotypes at the HEXA locus associated with infantile Tay-Sachs Disease. All B1 mutations which have been reported elsewhere have a substitution in codon 178 (exon 5) in which arginine is replaced by histidine (6), cysteine (7) or by leucine (4). Site-specific mutagenesis studies by Brown et al (8) have confirmed that Arg178 is required for the catalytic activity of Hex A. Patients 1 and 2 were previously reported to be heterozygous for a B1 phenotype (1). The fibroblast Hex A obtained from both these patients showed a profound decrease in the ratio of 4MUGS/4MUG hydrolysis which is a measure of catalysis by the enzyme's α-subunit relative to catalysis by its ß-subunit. Furthermore, residual catalytic activity toward 4MUGS had a pH optimum in the range of 2.0-2.6 compared to 3.9 for control Hex A. This unusual property in both patients suggested a common B1 allele which was different from either of the codon 178 B1 mutations. The aspartyl residue at codon 258 may, therefore, play a role in the catalytic mechanism of glycoside cleavage. The suggestion that aspartyl and/or glutamyl residues function in hexosaminyl bond cleavage has received some experimental support from the site-specific mutagenesis studies of Vavougios and Mahuran (9). We speculate that this aspartyl group furnishes a proton required for glycoside hydrolysis and in the mutant enzyme residual α-subunit catalytic function occurs at low pH because the only protons available are in water.

The initial genotype assignment of the parents of patient 1 and 2, based on serum Hex A and Hex B measurement have been proven correct by data presented in figure 2. The father of patient 1 and the mother of patient 2, both carrying the exon 7 mutant allele, had enzyme values within the TSD heterozygote range when genotypes were assigned using the 4MUGS procedure whereas the values were in the "grey zone" when Hex A and B were assayed with 4MUG using thermal fractionation (1) (%Hex A in serum: control range, 60-81%; heterozygote range, 34-55%; father patient 1, 57%; mother patient 2, 55%). Kinetic studies performed on Hex A prepared from fibroblasts obtained from patients 1 and 2 gave different Vmax values (expressed as a ratio of α/ß active sites). When expressed in this manner the Vmax is independent of the number of enzyme molecules and becomes equivalent to a turnover number. The Vmax of patient 1 Hex A was 0.29 and of patient 2 Hex A was 0.006 nmole 4MUGS cleaved/4MUG unit of enzyme. Control Hex A has a Vmax of 0.91 nmoles 4MUGS cleaved/4MUG unit of enzyme (1). Since the exon 8 mutation is incompatible with gene product expression the assignment of the B1 phenotype to the G772C allele becomes unequivocal. The relatively higher value for patient 1 Vmax suggested that the other TSD allele, carrying the exon 5 mutation, might be associated with a small but significant amount of kinetically normal Hex A. The exon 5 mutation is a hydrophilic to hydrophobic substitution, which is compatible with a conformation change in the protein which prevents exit from the rough endoplasmic reticulum (10,11). Nakano et al (12) have identified a TSD mutation at an adjacent base in codon 170. The occurrence of multiple mutations at this CpG dinucleotide pair suggests the presence of a mutagenic "hotspot" (13).

Both patients inherited the exon 7 mutation from a parent with Scottish-Irish heritage. The exon 5 mutation is of French-Canadian origin (family 7 in reference (14)). French Canadians have a frequency of TSD higher than that of the general population and the predominant mutation in this population group (15) is a 7.6 kb deletion at the 5' end of the HEXA gene (16). This mutation has a center of diffusion in the Gaspé region of the province of Québec (17). The exon 5 mutation has not been previously encountered in the province of Québec. The French Canadian ancestors of patient 1 emigrated from a region of Québec different from those known to be centers of diffusion for respectively, the common exon 1 mutation and the intron 7 +1 TSD allele (Lac St. Jean) (14). A second case of infantile TSD due to the exon 5 allele (R. Gravel, personal communication) in a child of French Canadian ancestry has been identified in a family unrelated to that of patient 1. Ancestors of both exon 5 TSD patients emigrated from the Estrie region of Quebec and lived within 60 miles of each other. It would be surprising if among the present inhabitants of this region additional carriers of the C508T TSD allele were not found.

3.6 ACKNOWLEDGEMENTS

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CHAPTER 4

A CHRONIC GM2 GANGLIOSIDOSIS VARIANT WITH A HEXA SPLICING DEFECT: QUANTITATION OF HEXA MRNA'S IN NORMAL AND MUTANT FIBROBLASTS

A chronic GM_2 gangliosidosis variant with a HEXA splicing defect: Quantitation of HEXA mRNA's in normal and mutant fibroblasts.

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4.1 ABSTRACT

Over 72 mutations have been identified in the *HEXA* gene of which, only three, G269S, K197T and Y180H, cause a chronic form of Tay-Sachs Disease (TSD). We identified a fourth chronic TSD mutation, IVS7,-7 G->A, in a Canadian patient of English ancestry. The second allele carries the common exon 11-4 bp insertion mutation which is the most frequent cause of TSD among Ashkenazi-Jews.

The IVS7, -7 G->A mutation reduces *HEXA* mRNA expression by 90%. Two mRNA species are produced from this allele; a normal species and one lacking exon 8. Although the IVS7, -7 G->A mutation introduces a new 3' acceptor site five base pairs upstream of the intron 7 acceptor site we found no mRNA species that was spliced at this new acceptor.

We used competitive PCR to quantitate the three mRNA species in fibroblasts obtained from this patient and compared these amounts to normal *HEXA* mRNA levels in cells from individuals who are homozygous normal, TSD heterozygotes and compound heterozygotes for the G269S allele which is also associated with a chronic TSD phenotype.

HEXA mRNA expression in normal fibroblasts was 17.3 pg/ug RNA. In individuals heterozygous for the exon 11-4 bp insertion 8.4 pg of normal *HEXA* mRNA was

produced /ug RNA. The *HEXA*, 1277 TATC, mRNA species was present in cells heterozygous for this mutation at 2.5% of the level of normal *HEXA* mRNA in homozygous normal cells.

In the fibroblasts from the chronic G_{M2} gangliosidosis patient heterozygous for the IVS 7, -7 G->A mutation the exon 8-deleted *HEXA* mRNA was 5.0% of that produced by homozygous normal cells and the normal *HEXA* mRNA species was 6% of normal. By contrast *HEXA* mRNA levels produced in cells compound heterozygous for the G269S TSD allele were normal.

4.2 INTRODUCTION

Tay-Sachs disease (TSD) is a neurodegenerative disorder characterized by the accumulation of G_{M2} ganglioside due to the deficiency of hexosaminidase A (Hex A). Hydrolysis of G_{M2} ganglioside is catalyzed at the α–subunit of this heterodimeric enzyme (αβ). Mutations at the *HEXA* gene, which encodes the α–subunit, cause TSD and those in HEXB, the gene encoding the β–subunit, are associated with Sandhoff Disease (SD) (reviewed in Gravel et al, 1995).

Classical infantile-onset Tay Sachs disease is caused by more than 40 different alleles at the *HEXA* gene which produce completely deficient or defective hexosaminidase A (Hex A) (reviews, Gravel et al. 1995; Hechtman and Kaplan, 1993). By contrast chronic or adult-onset forms of G_{M2} gangliosidosis, which are more clinically heterogeneous appear to be due, in almost all cases, to either homozygosity or compound heterozygosity for the G269S mutation (Navon and Proia, 1989; Paw et al. 1989). Two other Hex A mutations, K197T (Akli et al, 1993), and Y180H (De Gasperi et al, 1995), have been proposed to cause the chronic form of G_{M2} gangliosidosis.

A novel and unusual mutation, IVS7 -7 G->A, was identified in a chronic G_{M2} gangliosidosis patient whose clinical presentation has been previously reported (Parnes et al, 1985). The IVS7, -7 G->A allele is the first chronic TSD mutation known to affect splicing. The majority of splicing mutations in the *HEXA* gene cause infantile TSD and

only two, G570A (Akli et al, 1990) and IVS6 +1 G->A (Akli et al, 1993), cause the severe subacute form of the disease.

Since the patient is affected with a milder form of the disorder than is compatible with a complete absence of normally spliced mRNA, we characterized and quantitated the mRNA species in the patient's fibroblasts. We also quantitated the mRNA produced by the patient's second allele, the common 4 bp insertion (TATC) at position 1277 (exon 11) which is the most frequent mutation associated with the infantile-onset form of TSD (Myerowitz and Costigan, 1988).

Clinical heterogeneity has been observed among patients carrying the G269S mutation (Navon et al, 1986; Frederico et al, 1991). Although this mutation produces an unstable α–subunit (Brown and Mahuran, 1993) the affected nucleotide is the last in exon 7 and might, therefore play a role in correct mRNA splicing. We therefore investigated whether alternative or reduced splicing might also contribute to the enzymatic deficiency and whether variations in the response of the splicing machinery between individuals could explain their phenotypic difference.

4.3 MATERIALS AND METHODS

4.3.1 Cell lines WG1048 is the cultured fibroblast cell line obtained from the chronic TSD patient. WG1499 is from an infantile TSD patient homozygous for the exon 11-4bp insertion mutation. WG 1113, an obligate heterozygote for the exon 11-4 bp insertion mutation, was made available by Dr. M.M. Kaback of UCSD. WG 884 was obtained from a French-Canadian TSD patient who was homozygous for the 7.6 kb deletion mutation (Hechtman et al. 1990). WG1116 is from an adult TSD patient compound heterozygous for the G269S and +TATC 1277 mutations. MCH 39 is a normal control cell line. Fibroblasts were grown as previously reported (Hechtman, 1983)

4.3.2 Single Stranded Conformational Polymorphism Analysis Amplification of all *HEXA* exons, digestion of amplification products and electrophoresis was according to Triggs-Raine et al (1991).

4.3.3 DNA Sequencing Double stranded cycle sequencing was performed according to Sanger et al (1977) using the BRL kit.

4.3.4 RTPCR Total RNA was extracted from cells using the TrizolTM reagent (Gibco/BRL). The poly A+ content of fibroblast RNA was 5% as measured by oligo dT cellulose chromatography. RTPCR was performed using primers and conditions

described by Trop et al (1992) except that oligo dT20 was used as the primer for reverse transcriptase.

4.3.5 Diagnostic for the exon 11 4 bp insertion mutation This mutation was confirmed using allele-specific hybridization following amplification of exon 11 (Myerowitz and Costigan, 1988)

4.3.6 Quantitation of mRNA Standard cRNAs for the normal allele and each of the mutant alleles contained a 322 bp insert derived from the vector pUC 19. Therefore, in all the quantitation gels (figures 5 to 11) the upper band corresponds to the cRNA standard. Standards were constructed as follows: normal *HEXA* cDNA was cloned into the Not I/Xba I sites in the vector pRCCMV (Clontech) (pRCCMVαWT) or into the Bam H1/Xho I sites of pBluescript (pBSαWT). Constructs carrying the *HEXA* cDNA exon 8 deletion (pRCCMVα–exon8) were prepared by excision of a Ppu MI/Bst EII fragment of the plasmid pRCCMVαWT and replacement of this fragment with an exon 8-deleted cDNA fragment prepared by RTPCR of *HEXA* mRNA from cell line WG1048. A pBSα1277TATC construct was prepared by Bst EII/Sna BI digestion of the pBSαWT vector and replacement of this fragment with a 91bp fragment obtained by Bst EII/Sna BI digestion of the exon 11 amplification product (276 bp) of genomic DNA from the cell line WG1499. These three constructs (pRCCMVαWT, pBSα1277TATC, pRCCMVα–exon8) were used to prepare competitive PCR standards. All three plasmids were

linearized by digestion with Bst EII. A pUC 19 322 bp Pvu II restriction fragment was treated with the Klenow portion of DNA polymerase I to form a blunt ended fragment which was then ligated into the Bst EII site of the constructs within the cDNA insert resulting in pUC inserts in both sense and antisense orientations. In the antisense orientation there is a Xba I site in the insert in addition to the unique site in the vector. The use of the pRCCMV plasmids as templates for RNA polymerase depended upon linearizing the plasmid at this unique Xba I site which is immediately downstream of the insert. Selection of standard clones containing the pUC fragment in sense orientation was accomplished by screening for the presence of a single Xba I site. pRCCMV plasmids were linearized by Xba I digestion. The pBSα1277TATC construct was linearized with Not I. cRNA was synthesized from these templates using T3 RNA polymerase (Gibco/BRL) for the pBS construct or T7 RNA polymerase for the pRCCMV constructs and products were purified using oligo-dT-cellulose. The cRNA standards were quantitated spectrophotometrically using the extinction coefficient 1 OD260 = 40 ug RNA/ml.

Competitive PCR was performed according to Scheuermann and Bauer (1993) and intensity of cDNA fragments produced by mixtures of patient total RNA and standard RNA was estimated visually following electrophoresis in 1.4% agarose and staining with ethidium bromide.

The following primers were used for competitive PCR: primer 1 (5'), CCTGGATTACTGACTCCTTG; primer 2 (3'), AGGGGTTCCACTACGTAGAA; primer3(3'),GTCAGGGCCATAGGATAGAT;primer4(5')TTGTCCTGGGGACCAGGA AG and primer 5 (5')AACCCAGAGATCGAGGACTT. Primers 1 and 2 amplify normal *HEXA* mRNA, the mutant mRNA species carrying the exon 11-4 bp insertion and the mRNA containing the G269S mutation. Primers 5 and 3 amplify only *HEXA* mRNA containing the exon 11-4 bp insertion. Primers 2 and 4 amplify only a species of mRNA in which exon 8 is deleted.

PCR amplification conditions used were: for primer pairs 1/2 and 2/4; 1 min @ 94C start followed by 30 cycles of 1 min @ 94C, 1 min @ 55C and 3 min @ 72C and for primers 5 and 3; 30 sec @ 94C start followed by 32 cycles of 30 sec @ 94C, 30 sec @ 60C and 90 sec @ 72C.

4.4 RESULTS

4.4.1 Identification of the Novel Mutation SSCP analysis of all 14 exons showed aberantly migrating electrophoretic bands only for the Alu I digested products of exon 8 (Fig 1). Direct sequencing of amplified exon 8 (Fig 2) in patient DNA identified heterozygosity for a G->A substitution in the -7 position of IVS 7. The mutation was confirmed by Alu I digestion of amplified exon 8. Figure 3 shows that, in addition to the digest fragments of 162 and 107bp found in control DNA, the patient DNA has an additional restriction site resulting in the appearance of a 126 bp fragment.

The second mutation was determined by allele-specific hybridization to be the common exon 11-4 bp insertion (data not shown).

4.4.2 Quantitation of *HEXA* mRNA species The amplification of the normal *HEXA* cDNA using primer pairs flanking exon 8 (Trop et al. 1992; Akalin et al, 1992) yields a product of 392 bp (Fig 4, lane N). Faint signals at 392 and 211 bp are apparent (lane P) when RTPCR is performed using the same primer pair with mRNA from WG1048 fibroblasts. The difference between the two fragments (181bp) corresponds to the size of exon 8 and shows that one of the consequences of the IVS 7,-7 G->A mutation is the skipping of exon 8. This deletion from a *HEXA* mRNA results in a frameshift downstream from the exon 7-9 junction.

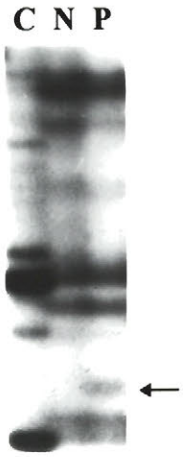

Figure 1 Single stranded conformational polymorphism analysis of amplified, Alu I digested, exon 8 genomic DNA. Lane C, undenatured normal control DNA. Lane N, denatured normal control DNA. Lane P, denatured patient DNA. The arrow indicates the aberrantly migrating band.

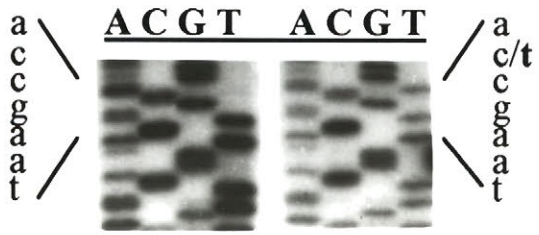

Figure 2 Direct sequencing, of the antisense strand, of exon 8 amplified from normal (left) and patient (right) genomic DNA.

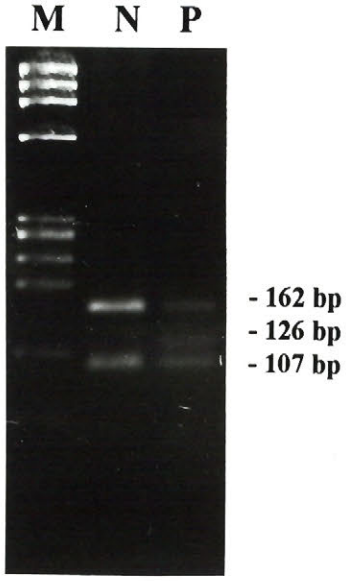

Figure 3 Restriction analysis of amplified exon 8 genomic DNA. Lane M, molecular weight marker. Lane N, Alu I digestion of control DNA. Lane P, Alu I digestion of patient DNA.

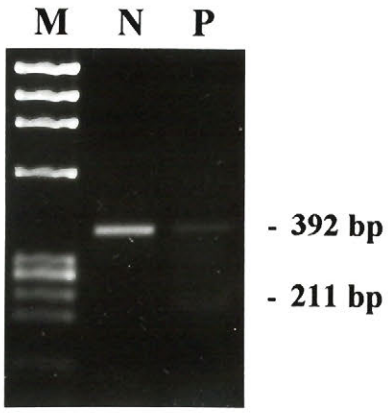

Figure 4 RTPCR of fibroblast mRNA. Lane M, molecular weight markers. Lane N, normal control mRNA. Lane P, patient mRNA

Both RTPCR amplification products from WG1048 mRNA were recovered and directly sequenced (data not shown). As predicted, the 211 bp product showed a loss of exon 8 sequence. The 392 bp fragment showed only a single normal mRNA species with no intron sequences between exon 7 and exon 8 indicating that the new 3' acceptor site in intron 7 created by the mutation is not used by the splicing machinery.

The primers used for competitive PCR were tested for their specificity using a mRNA preparation from fibroblasts obtained from a homozygous normal individual (expressing only normal *HEXA* mRNA) and from fibroblasts obtained from an individual homozygous for the French-Canadian TSD deletion mutation which are expected to produce no *HEXA* mRNA.

Figure 5 shows the results of competitive amplification of a constant amount of MCH39 mRNA using primers 1 and 2 in the presence of variable amounts of the cRNA standard. The point at which amplication products of the mRNA and cRNA appear at equivalent intensity corresponds to the expression, in normal fibroblasts of 17.3 pg *HEXA* mRNA/ug total mRNA (Table 1). In all figures, the values marked above each lane correspond to the amount of cRNA used and the corrected value corresponding to the amount of patient mRNA is shown in Table 1. Lanes marked "pr 5&3" and "pr 2&4" in which no amplification products are seen show, respectively, the results of substitution of primers 1 and 2 by primers 5 and 3 (which amplify the exon 11-4 bp insertion mutation) and

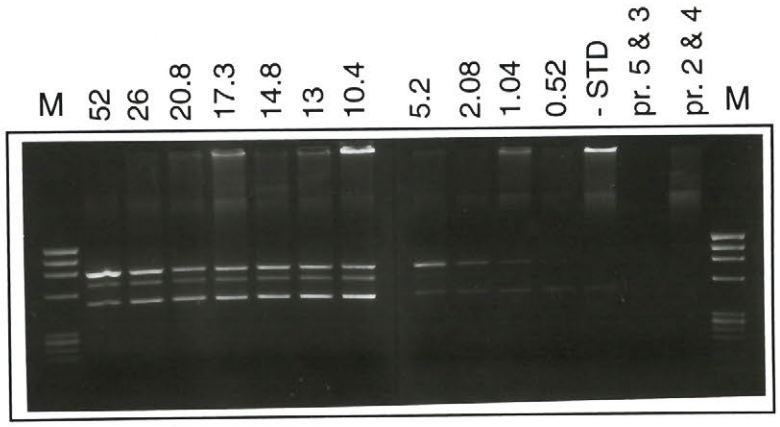

Figure 5 Quantitative determination of normal HEXA mRNA in homozygous normal fibroblasts. Lanes M, molecular weight markers, Hae III digest of ϕX174 DNA. Lanes 52 - 0.52 indicate pg of normal standard used in each lane for competitive PCR. STD, amplification of normal standard alone. pr. 5 & 3 and pr. 2 & 4, substitution of the indicated primer pairs for primer 1 & 2.

Table I

HEXA mRNA levels in human fibroblasts

Cell Line	Genotype	Normal	1277_{TATC} exon 8	
MCH 39	+\+	17.3 [1]	ND [2]	ND
WG1113	+\$1277_{TATC}$	8.4	0.5	-
WG884	7.6kb \ 7.6kb	<0.05	-	-
WG1048	1277_{TATC}/ IVS7,-7 G->A	1.6	0.5	1.0
WG1116	G269S/ TATC (1277)	11.3 [3]	0.5	

1. Values are reported as pg HEXA mRNA/ug total cellular RNA

2. Not Detectable

3. mRNA with G269S mutation

substitution by primers 2 and 4 (which amplify only the mRNA in which exon 8 is deleted).

The intermediate band migrating between the mRNA and cRNA product was shown to be a heteroduplex of strands of the two amplication products (Figure 11, lane 5). This band does not occur when either cRNA (lane 1) or mRNA (lane 2) are amplified and electrophoresed separately or when the RTPCR mixtures in which cellular mRNA and control cRNA were amplified separately and co-electrophoresed (lane 3). When the two RTPCR reactions were mixed following separate amplifications, and heated and slowly cooled prior to electrophoresis, the intermediate band is seen. Mung bean nuclease, which digests only single stranded nucleic acids, abolishes the intermediate band but does not affect the two homoduplex amplification products (data not shown).

RTPCR amplification of normal *HEXA* mRNA sequences from the fibroblast cell line homozygous for the 7.6 kb deletion mutation using primer pair 1 and 2 showed a complete absence of product (<52 fg/ug RNA (data not shown) confirming that the deletion mutation is not compatible with expression of *HEXA* mRNA. Figures 6 and 7 show the results of measurement of normal and mutant mRNA species in the fibroblasts of cells from an obligate TSD heterozygote carrying the exon 11-4 bp insertion mutation. The values obtained for these species were, respectively, 8.4 and 0.53 pg/ug RNA. Thus the TSD heterozygote, who expresses approximately half of the normal amount of Hex A enzyme, also expresses half the amount of *HEXA* mRNA as the normal homozygote.

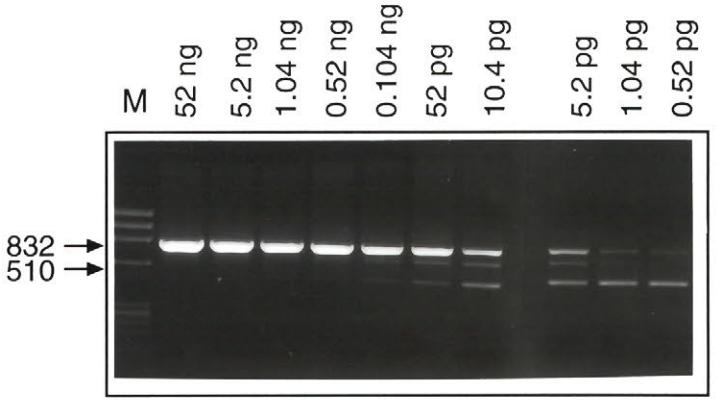

Figure 6 Quantitative determination of normal HEXA mRNA in fibroblasts heterozygous for 1277_{TATC} mutation (WG1113). The amount of the normal standard in ng or pg is indicated above each lane.

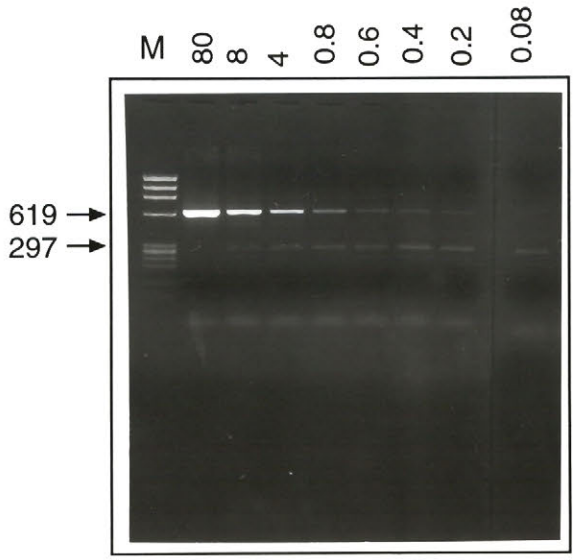

Figure 7 Quantitative determination of HEXA mRNA carrying the 1277_{TATC} mutation in fibroblasts heterozygous for this mutation (WG1113). The amount of standard cRNA carrying the 1277_{TATC} mutation is indicated in pg for each lane.

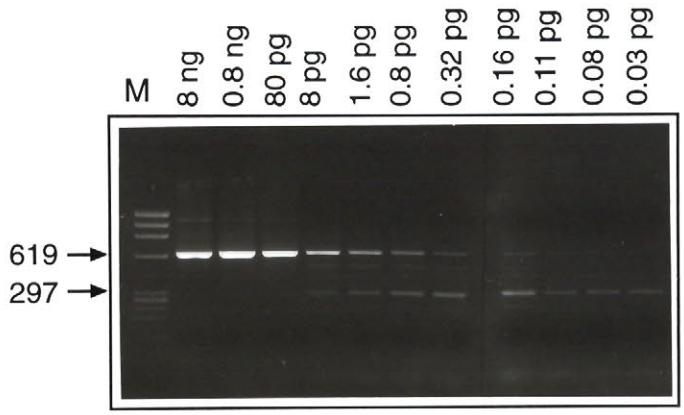

Figure 8 Quantitative determination of HEXA mRNA carrying the 1277_{TATC} mutation in WG1048 fibroblasts. The amount of standard cRNA carrying the 1277_{TATC} mutation is indicated for each lane.

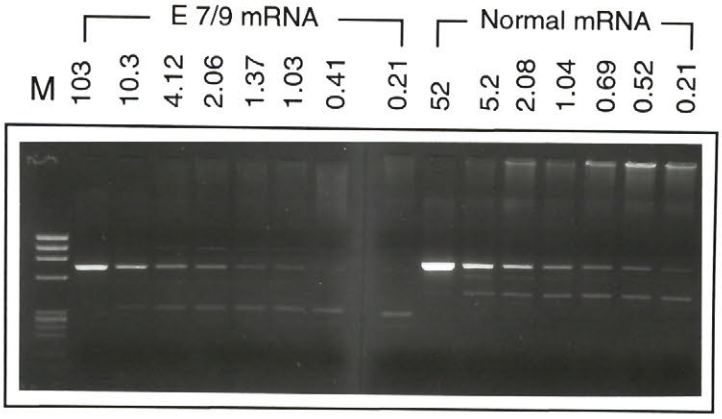

Figure 9 Quantitative determination of exon 8 deleted mRNA (left) and normally spliced mRNA (right) in WG1048 cells. Amount of appropriate standard cRNAs in pg are indicated for each lane.

Since primers 1 and 2 amplify both normal mRNA and mRNA carrying +TATC (1277) mutation, the "normal" value obtained from the heterozygote cell line will also include the amount of mutant mRNA detected using the more specific amplification primers 5 and 3.

Figures 8 shows the quantitative estimation of the +TATC (1277) mutation in an RNA preparation from WG1048 cells and Figure 9 shows the quantitative estimation of the "normal" *HEXA* mRNA species and the *HEXA* mRNA species in which exon 8 is deleted. As in the case of the heterozygote, the "normal" *HEXA* mRNA value will also include the product of amplification of mRNA containing the insertion mutation (Table 1).

Figure 10 shows the quantitative determination of the mRNA species carrying the G269S mutation in WG1116 fibroblasts. This mutation appears to be compatible with a normal level of expression of mRNA (Table 1).

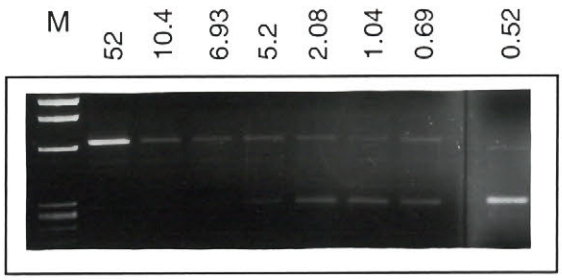

Figure 10 Quantitative determination of HEXA mRNA carrying the G269S mutation in WG1116 fibroblasts. The amount of standard cRNA used is indicated for each lane.

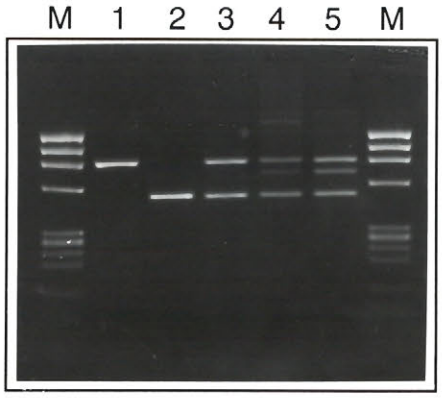

Figure 11 Appearance of heteroduplex band during competitive PCR. Lanes M, molecular weight markers. Lane 1, amplification of mRNA template. Lane 2, amplification of cRNA template. Lane 3, amplification of mRNA and cRNA templates separately and coelectrophoresis. Lane 4, amplfication of mRNA and cRNA templates separately. Amplified products were heated together and slowly cooled before electrophoresis. Lane 5, competitive amplification of mRNA with cRNA standard.

4.5 DISCUSSION

The majority of splicing mutations in *HEXA*, as well as in other genes, (Krawczak et al, 1992; Gravel, 1995) occur in the invariant GT at the 5' donor end of the intron. Acceptor site mutations (Mules et al, 1991; Tanaka et al, 1994) are much less frequent.

We have characterized a novel acceptor splice site mutation in the *HEXA* gene that is associated with the chronic form of TSD. This mutation, IVS7, -7 G->A, creates a new invariant 3' intron acceptor site, by virtue of the surrounding sequence, five base pairs upstream of the normal site and reproduces an identical copy of the last five bases in intron 7. The splicing alternatives for this allele include i) preferential use of the new site by the RNA splicing machinery, ii) preferential use of the normal acceptor site, iii) skipping of the adjacent exon to produce a mRNA deleted in exon 8, iv) reduction in splice products or v) some combination of these alternatives.

The major observed effect of this mutation was the reduction of cytoplasmic *HEXA* mRNA by >90% with the residual mRNA comprising two species; the normal *HEXA* mRNA (at 6% of normal levels) and a mRNA species lacking exon 8 (5% of normal). The latter mRNA has an out of frame deletion resulting in the synthesis (if any) of an inactive protein. No mRNA species containing the last five bases of intron 7 was detected indicating that the new 3' acceptor site was not utilized.

Exon skipping at the IVS 7, -7 G->A allele may be explained by the splicing model proposed by Robberson et al (1990). Pre-mRNA splicing involves cleavage at the 5' splice site yielding a 'free' 5'-exon and lariat intermediate. The RNA is then cleaved at the 3' splice site followed by the concomitant joining of the two exons. Since the intermediate state (lariat structure and 5'-exon) consists of two RNA's that are efficiently converted to the final product, it was suggested that these RNA's remain bound in a complex. This complex has been identified and is known as the splicing body or spliceosome. The spliceosome is a large RNA-protein complex that catalyses the removal of introns from nuclear pre-mRNA. It is comprised of three major RNA-protein subunits, the U1, U2 and U4/U6, U5 small nuclear ribonucleoprotein particles (snRNP's) and an additional group of non-snRNP protein splicing factors. According to Robberson, the binding of factor U2 to the 3' acceptor site of the intron is the event that initiates splicing. If it is hypothesized that the IVS 7, -7 G->A mutation reduces binding of U2 to the normal acceptor site, then this mutation would cause the splicing machinery to search for a consensus acceptor site further downstream causing exon skipping.

The level of *HEXA* mRNA/ug total RNA in normal cells (17.3 pg) corresponds to 0.035% (omitting size variation among mRNA species) of the cellular mRNA population. Estimates of the Hex A content of tissues based on enzyme purification studies give percentages for this enzyme in the same range (Verpoorte, 1972; Mahuran and Lowden, 1980; Srivastava et al. 1974; Geiger and Arnon, 1976; Sandhoff and Waessle, 1971). *HEXA* mRNA in homozygotes for the French-Canadian deletion mutation was

undetectable. Although this result is expected since this mutation involves the loss of both exon 1 and upstream promoter sequences, it serves as a validation of quantitation experiments by demonstrating that our primers are not amplifying mRNA species transcribed from other genes. *HEXA* mRNA with the +TATC (1277) insertion mutation was estimated in both the chronic TSD patient and in fibroblasts obtained from a normal heterozygote. The steady-state level of this species of *HEXA* mRNA in both cell lines is about 2.5% of normal *HEXA* mRNA expression in homozyous normal fibroblasts. Previous attempts to detect this species of *HEXA* mRNA using northern blotting of RNA preparations from fibroblasts homozygous for this mutation were not successful (Myerowitz et al. 1985), however primary transcripts of this gene were later demonstrated in the same cells using "nuclear run on" assays" (Paw and Neufeld, 1988). This species of mRNA is also seen in COS cells transfected with αcDNA carrying the +TATC (1277) mutation (Nishimoto et al., 1991).

It has been suggested that in mRNAs in which premature stop codons are associated with rapid turnover, the mutation may cause a change in secondary structure which unmasks a digestion site for RNAase (Zhang et al, 1994). A minigene construct containing the four base pair insertion mutation and a single base pair deletion to correct the frameshift error, restored *HEXA* mRNA levels (Boles and Proia, 1995). These results clearly demonstrate that the instability of the mRNA is due to the premature stop codon and not the mutation itself.

Heteroallelism is often associated with a wide variation in phenotype. The G_{M2} gangliosidoses, many of which are caused by *HEXA* mutations, include infantile, juvenile and adult onset TSD as well as multiple neurological phenotypes such as dystonia (Meek et al. 1984), ataxia (Willner et al. 1981; Harding et al. 1987; Johnson et al. 1977) or motor neuron disease (Federico, 1987; Rubin et al. 1988; Mondelli et al. 1989; Streifler et al. 1989) with or without psychiatric disorders. The biochemical basis for these phenotypic differences is not known but available evidence supports the hypothesis that the severity of the disorder is inversely related to the amount of residual Hex A activity associated with the patient's genotype. Such correlations are not easily established for the less severe variants especially when variable phenotypes are observed in patients with the same genotype.

Mc Innes et al (1992) proposed that individual differences in splicing pathway preferences could explain such "nongenetic" clinical variation. Since the G269S mutation occurs within the splice consensus region it was predicted that reduced splicing could contribute to the phenotype and variation in intron 7 splicing could explain the phenotypic heterogeneity seen in patients with this mutation. Our results suggest, however, that alternative splicing cannot explain the phenotypic heterogeneity associated with the G269S chronic TSD allele. In the case of the IVS7, -7 G->A mutation, however, competitive PCR is sufficiently sensitive and accurate to permit detection of low levels of normal mRNA expression sufficient to produce a mitigated phenotype.

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Connecting text - Chapter 4 to Chapter 5:

The identification of the putative active site mutation D258H, reported in chapter 3, and the lack of a sufficiently sensitive heterologous system for the study of TSD mutations led to the development of a novel expression system in a neuroglial cell line. This system was also used to study three putative active site residues identified on the basis of homology with other glycosyl hydrolases and crystallographic data.

Chapter 5 therefore addresses the last two objectives of this thesis, i) to establish a new expression system to study TSD alleles and ii) to identify putative active site residues in the α-subunit of *HEXA*.

CHAPTER 5

A TRANSIENT EXPRESSION SYSTEM FOR TAY-SACHS DISEASE GENES IN CULTURED NEUROGLIAL CELLS: EXPRESSION OF ACTIVE SITE MUTATIONS.

A transient expression system for Tay-Sachs disease genes in cultured neuroglial cells: Expression of active site mutations.

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5.1 ABSTRACT

Mutations in the *HEXA* gene, which encodes the α–subunit of the heterodimeric enzyme hexosaminidase A (Hex A), cause Tay-Sachs disease (TSD), an autosomal recessively inherited lysosomal storage disorder. The evaluation of the biochemical phenotype associated with *HEXA* mutations has been hampered by an insufficiently sensitive system for analysis of mutant hexosaminidases with varying amounts of residual enzyme activity. The major disadvantage of the heterologous gene expression system, the COS cell, has been the high-level of endogenous Hex A activity.

We report a novel transient expression system, in an SV40-transformed human fetal TSD neuroglial (NG) cell line which displays very low endogenous Hex A activity. Transfection of NG cells with *HEXA* cDNA resulted in 100-500 fold increase in 4MUGS hydrolysis (Hex A plus Hex S) relative to mock transfected cells. The virtual absence of enzyme activity in mock transfected cells permits comparative analysis of expression of mutations leading to variant clinical forms of G_{M2} gangliosidosis under conditions where residual enzymatic activity reflects the clinical severity of the disorder.

The TSD-NG cells were also used to test hypotheses regarding the catalytic role of putative active site residues at positions 258, 307, 323 and 462 in the α–subunit of Hex A. Mutant *HEXA* cDNAs carrying isosteric or isofunctional substitutions of the dicarboxylic acid residues at these positions in the α–subunit of human Hex A were expressed in NG cells.

Both αE323D, and αE462D cDNAs produced peptide chains that were normally processed from the 67kD precursor form to the 54kD mature (lysosomal) form but had drastically reduced activity toward the synthetic Hex A–specific substrate 4MUGS. The αE307D cDNA retained significant catalytic activity but the mutation prevented maturation of the α–subunit precursor. By contrast, the αD258N mutation produced an enzyme with drastically reduced enzymatic activity that matured at a rate greater than normal.

Our data suggest that E323 and E462 are active site residues and that E323 is involved in catalysis.

5.2 INTRODUCTION

The G_{M2} gangliosidoses are a group of ganglioside storage disorders characterized by the pathological accumulation of G_{M2} ganglioside in neuronal cells. The most common of these disorders, Tay-Sachs disease (TSD) results from a deficiency of hexosaminidase A (Hex A). Hex A is a heterodimer composed of an α–subunit (*HEXA* gene, chromosome 15q23-24) and a β–subunit (HEXB gene, chromosome 5q13-14). Hex B, a related hexosaminidase isoenzyme, is composed of two β–subunits. Hex S, a dimer of α–subunits, forms only under conditions of excessive α–subunit expression (1).

Heterologous transient expression systems have often been applied to determine the effect on enzymatic activity of gene mutations identified in the investigation of patients with hereditary metabolic disorders. Evaluation of the biochemical phenotype associated with a particular mutant allele depends upon the availability of appropriate gene expression systems. Gene expression systems can also be used to test hypotheses regarding the functional roles of amino acids or putative functional domains of a protein. We report (i) the development of a novel specialized transient expression system for the study of mutations in the *HEXA* gene which cause Tay-Sachs disease (TSD) (2) and (ii) the identification of putative active site residues of the α-subunit of Hex A through site-directed mutagenesis expression analysis.

HEXA alleles were expressed in an SV-40 transformed neuroglial cell line obtained from a TSD patient (3,4,5) using a vector driven by the cytomegalovirus (CMV) promoter to achieve very high levels of *HEXA* expression. There are several advantages to this system: i) a high level of gene expression, ii) no endogenous Hex A activity in untransfected cells permitting the analysis low levels of enzyme activity and iii) the expression of transfected *HEXA* genes as the physiological dimer Hex A.

The NG system was also used to test candidate active site residues of the α-subunit of Hex A. Potential active site residues, D258, E307, E323 and E462, were chosen on the basis of (i) TSD causing mutations with the B1 biochemical phenotype (6,7), (ii) invariant residues in family 20 of glycosyl hydrolases (8,9) and (iii) homology modelling with the chitobiase from *Serratia marcescens*, the sole member of family 20 that has been crystallized (10).

5.3 METHODS

5.3.1 Cell culture NG 125 and NG 141, SV40-transformed fetal, TSD and normal neuroglial cell lines respectively, were provided by L. Hoffman (Kingsbrook Jewish Medical Center, Brooklyn, NY). The cells were cultured in α–MEM supplemented with 15% fetal calf serum and antibiotics (antibiotic-antimycotic mix from BRL).

5.3.2 Recombinant plasmids The ß-galactosidase gene, in pSVLßgal (Clontech), was used as a reporter gene to control for transfection efficiency. Cloned human *HEXA* cDNA (11) was inserted into pRCCMV (Invitrogen) and pREP4 (Invitrogen) to produce pRCCMVα and pREP4α. Preparation of pRCCMVα involved; 1) subcloning *HEXA* from pSVLα into a pCRII (Invitrogen) derivative as a XhoI/ BamHI fragment, 2) subcloning *HEXA* into the PstI site of pBS (Stratagene) by digestion with NsiI, 3) subcloning a XhoI/ Xba I fragment from pBS into the pCRII derivative and 4) cloning a NotI/ XbaI HEXA cDNA containing fragment from pCRII into pRCCMV. pREP4α involved cloning the *HEXA* cDNA as a XhoI/BamHI fragment from pSVLα into pREP4.

pCMVα was constructed by creating a *HEXA* cDNA flanked by Not I sites. The *HEXA* cDNA insert in the pRCCMVα plasmid already has a Not I site at the 5' end of the gene. The insert was subcloned into the BamHI site of pBS (KS^-) (Stratagene) introducing a second Not I site downstream from the 3' end of the gene. This step permitted the cloning

of the insert into the unique Not I site of pCMV after removing the *E. coli* ß-galactosidase gene from pCMVß (Clontech) to create pCMVα. All plasmids were purified by Qiagen column chromatography prior to transfection.

5.3.3 Site-directed mutagenesis Mutations were introduced into the *HEXA* cDNA using a modified protocol of the Clontech Transformer Mutagenesis Kit. The second screening step for mutant clones was omitted. Mutant clones were identified after the first screen by PCR amplification of samples of isolated bacterial colonies followed by restriction enzyme digestion or allele-specific hybridization of amplified product to identify mutant genotypes. Mutations were introduced into pSVLα and a cassette containing the altered sequence was subcloned into pBS and the full length mutant cDNA subsequently subcloned into pCMV. Mutant pCMVα inserts were sequenced (Pharmacia T7 Sequencing Kit) and the plasmids purified by Qiagen column chromatography prior to transfection.

5.3.4 Transfection For transient expression in cell line NG 125, subconfluent T175s (175 cm^2, Sarstedt) were harvested by trypsinization and washed twice with 1X PBS. The cell pellet was resuspended in Optimem medium (BRL, containing 5% FBS) to obtain a final concentration of $6X10^6$ cells/ml. Cell suspension (800ul), 20ug pCMVα and 2ug pSVLßgal were placed in a 0.4 cm cuvette, mixed, placed on ice for 5 minutes and pulsed (500uFd, 400V) using a Biorad electroporation appartus. The time constant was between 16 and 18 msec. After 15min on ice 800ul α–MEM (without antibiotics) was added to the

suspension. For the ß-galactosidase qualitative assay 250ul of transfected cell suspension was plated in a 12 multiwell plate. The remainder of the transfected cell suspension was grown for 48 hours in a T75 (75 cm^2).

5.3.5 Enzyme and protein assays A qualitative ß-galactosidase assay was performed to determine the percentage of surviving cells which expressed the bacterial enzyme. The multiwell plated-cells were incubated as described by Lake (12). After 24h incubations the number of blue cells was estimated microscopically. Harvested cells were lysed by freeze-thawing in 0.25M Tris-HCl (pH7.4) and protein determined by the Bradford method (Biorad). A fluorescent assay (13) was adapted for quantitation of ß-galactosidase activity in transfected cell lysates. The reaction mixture contained: 3ul 100X Mg solution (4.5 M 2-mercaptoethanol, 0.1 M $MgCl_2$), 100ul 0.5mM 4MUßgal and approximately 2 to 5ug of lysate protein in 0.1M sodium phosphate buffer (pH7.5) in a volume of 334ul. After incubation at 37°C for 15 minutes fluorescence, due to release of 4MU, was determined using a Perkin Elmer spectrofluorimeter (excitation wavelength: 360 nm, emmission: 447 nm). Hexosaminidase activity was also determined fluorometrically using either 4MUGS (Hex A–specific substrate) (14) or the 4MUG (Hex A and B) substrate.

5.3.6 Western blot analysis The enhanced chemiluminescence (ECL) Western blotting kit from Amersham was used to detect the presence of the Hex A α–subunit with a polyclonal rabbit anti-human Hex A antibody. Both the primary and secondary (rabbit Ig, horseradish peroxidase-labelled antibody) antibodies were used at a 1:5000 dilution.

5.3.7 Chromatofocusing of transfected cell extracts The hexosaminidase isoenzyme profile was determined by chromatofocusing using the Pharmacia Polybuffer Exchanger (PEB) system. The protocol of O'Dowd et al (15) was modified to analyse a quarter of the amount of protein. Transfected cell extracts were freeze-thawed (3X) in 0.025M imidazole buffer and protein concentration determined by the Bradford method. Approximately 1ml of PEB74 gel was used to prepare a column in a 1 ml syringe. The column was washed with the equivalent of 1.5-2X the bed volume with 0.025M imidazole (pH 7.4). The protein extract was added after passing 2X 100ul of Polybuffer (pH 4.0) through the column. Four hundred microliter fractions were collected. To elute the Hex S isoenzyme, 0.13M sodium citrate buffer (pH 3.46) was run through the column after the eluant had attained a pH of 4.0 for several fractions (usually at fraction 28). Twenty microliters of each fraction was assayed for 4MUG and 4MUGS activity as described above and the pH of each fraction was determined.

5.4 RESULTS

5.4.1 Expression of the *HEXA* gene in NG cells Endogenous 4MUGS activity in NG 141 cells (normal neuroglial) was 570 nmole/mg/hr. In untransfected NG 125 cells the rate of 4MUGS hydrolysis is <1% of that in NG141 cells. This activity is probably due to the action of Hex B on 4MUGS (14).

Vectors carrying the *HEXA* gene were assessed for their ability to express enzymatic activity when transfected into NG125. Hexosaminidase activity in cells transfected with 20ug of each vector (pSVLα, pRCCMVα, pREP4α or pCMVα) was at least 10 fold higher than activity in mock transfected cells. The highest level of expression (approximately 1000-fold above mock-transfected controls) was achieved when cells were transfected with the pCMVα vector (Table 1A).

Analysis of hexosaminidase activity at 24, 48 and 72 hours post-transfection, indicated that hexosaminidase activity continued to increase up to 72 hours (Table 1B).

Western analysis of transfected (20ug pCMVα) and mock (transfection with only pSVLßgal) cell extracts confirmed that the increase in 4MUGS activity in TSD-NG cells is due to α–subunit expression (Figure 1). Both the precursor and mature α–subunit are absent in mock cells but present in cells transfected with pCMV α.

5.4.2 The Hex isoenzyme profile in transfected NG cells To determine whether the α–subunits encoded by the *HEXA* gene were expressed as the heterodimeric enzyme Hex A (αß) or the homodimeric species Hex S (α_2) or both, transfected NG cell lysates were chromatofocused using the PEB system of Pharmacia. Figure 4 illustrates the chromatofocusing profile of cells transfected with the normal pCMVα vector. All three hexosaminidase isoenzymes were present, well separated and eluted at their approximate pI's. Hex S is the isoenzyme present in greatest abundance followed by Hex A and Hex B respectively.

5.4.3 Expression of *HEXA* mutations Three *HEXA* mutations (G269S, G250D, R170W) associated with the adult, juvenile and infantile-onset forms of TSD respectively, were expressed in NG-125 cells. The expression of the G250D and G269S mutations was analyzed in this system and compared to the expression of these same mutations in COS. Expression of these two mutations resulted in 4-fold and 9-fold greater 4MUGS activity respectively than in mock transfected cells (Table 2). In contrast, R170W exhibited no significant level of 4MUGS activity above mock transfected cells (Table 2). Western blot analysis revealed that the α–subunit synthesized by all three mutant plasmids appeared in the precursor form. The G269S mutant was compatible with the expression of a small amount of mature α–subunit (Figure 1). These results are in agreement with what is known about the G269S (16) and G250D (17) mutations and confirm the association of the R170W mutation with the infantile TSD phenotype.

Expression of *HEXA* in Neuroglial Cells

A. Comparison of *HEXA* expression vectors

Cell line	Vector	Hours[1]	Hex Specific Activity[2]
NG-125	-	48	4
NG-125	pSVLα	48	43
NG-125	pRCCMVα	48	358
NG-125	pREP4α	48	611
NG-125	pCMVα	48	3986

B. Expression of *HEXA* in NG cells over time

Cell line	Vector	Hours[1]	Hex Specific Activity[2]
NG-125	pCMVα	24	1825
NG-125	pCMVα	48	3986
NG-125	pCMVα	72	7145

1. hours harvested post-transfection

2. nanomoles 4MUGS hydrolyzed/h/mg protein

Table 1. A) NG125 cells were transfected with 20ug of each plasmid, harvested at 48 hours and 4MUGS activity determined. B) NG125 cells were transfected with 20ug of pCMVα, harvested at 24, 48 and 72 hours and 4MUGS activity determined.

Expression of G_{M2} gangliosidosis mutations

Genotype	Clinical Phenotype	Hex/ßgal Ratio
Wild type	**Normal**	**49.00 (+/- 15)**
G269S	**Adult**	**1.5 (+/- 0.8)**
G250D	**Juvenile**	**0.67 (+/- 0.3)**
R170W	**Infantile**	**0.20 (+/- 0.08)**
Mock		**0.16 (+/- 0.02)**

Table 2. NG125 cells were transfected with wild-type (pCMVα) or mutant (pCMVG269S, G250D or R170W) constructs. Cells were harvested after 48 hours. Transfection efficiency was corrected for by expressing hexosaminidase activity as a ratio of 4MUGS/βgal activity.

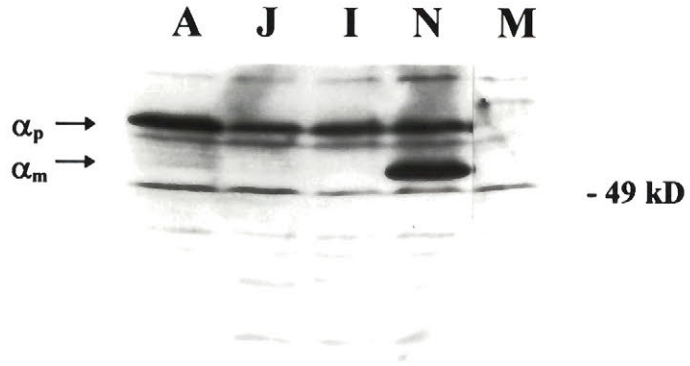

Figure 1. Western blot analysis of NG cells transfected with pCMVG269S (A), pCMVG250D (J), pCMVR170W (I) and pCMVα (N). Lane M represents mock transfected cells. The arrows indicate the position of the precursor (α_p) and mature (α_m) α–subunit. The other bands are background bands.

To assess the possible role of D258, E307, E323 and E462 in the Hex A active site the following mutant constructs were expressed in TSD-NG cells; D258H, D258E, D258N, E307D, E323D and E462D. The substitution D258H occurs in patients with the infantile TSD B1 phenotype (7, 18). Of the three codon 258 mutations only expression of D258N resulted in detectable levels of Hex A activity (3-fold higher than Hex A activity in mock-transfected controls) (Table 3). Western blot analysis of transfected cell extracts (Figure 2) revealed that the processing of all 258 mutants was affected. The most profound effect was observed on the maturation of the αD258H precursor and to a lesser extent on that of the D258E mutant. In contrast, αD258N precursor maturation seemed to occur more efficiently resulting in normal levels of mature α–subunit. Chromatofocusing of D258N cell extracts revealed that this mutation affected both 4MUG and 4MUGS hydrolysis (Hex S fractions).

The mutations E307D, E323D and E462D had a dramatic effect on catalytic activity (Table 4). E323D did not exhibit significant 4MUGS hydrolysis in comparison to the mock transfected cells. E462D and E307D, in contrast, exhibited 14-fold and 60-fold 4MUGS activity above mock. Only the 323 and 462 mutants synthesized equivalent amounts of precursor and mature α–subunit (Figure 3). Chromatofocusing of E323D and E462D cell extracts confirmed that the isoenzyme Hex A is processed normally but has a catalytically defective α–subunit that is unable to hydrolyze 4MUG and 4MUGS (Figure 4). In contrast, the E307D mutation dramatically affects α–subunit maturation. The mature form is only seen on overexposing the film (data not shown).

Effect of Codon 258 Substitutions on Hex A activity

Residue 258	Hex/ß/gal Ratio
D258	192 (+/- 32)
D258H	0.15 (+/- 0.04)
D258E	0.15 (+/- 0.05)
D258N	0.37 (+/- 0.06)
Mock	0.13 (+/- 0.07)

Table 3. NG125 cells were transfected with wild-type (pCMVα) or mutant (pCMVD258H, D258E or D258N) constructs. Cells were harvested after 48 hours. Transfection efficiency was corrected for by expressing hexosaminidase activity as a ratio of 4MUGS/βgal activity.

Figure 2. Western blot analysis of NG cells transfected with pCMVD258H, pCMVD258E, pCMVD258N and pCMVα (N). Lane M represents mock transfected cells. The arrows indicate the position of the precursor (α_p) and mature (α_m) α–subunit. The other bands are background bands.

Expression of E307, E323 and E462 mutants

Mutant	Hex/ß/gal Ratio
Wild-type	36 (+ 16)
E307D	3.6 (+/- 0.5)
E323D	0.2 (+/- 0.09)
E462D	0.85 (+/- 0.13)
Mock	0.06

Table 4. NG125 cells were transfected with wild-type (pCMVα) or mutant (pCMVE307D, E323D or E462D) constructs. Cells were harvested after 48 hours. Transfection efficiency was corrected for by expressing hexosaminidase activity as a ratio of 4MUGS/βgal activity.

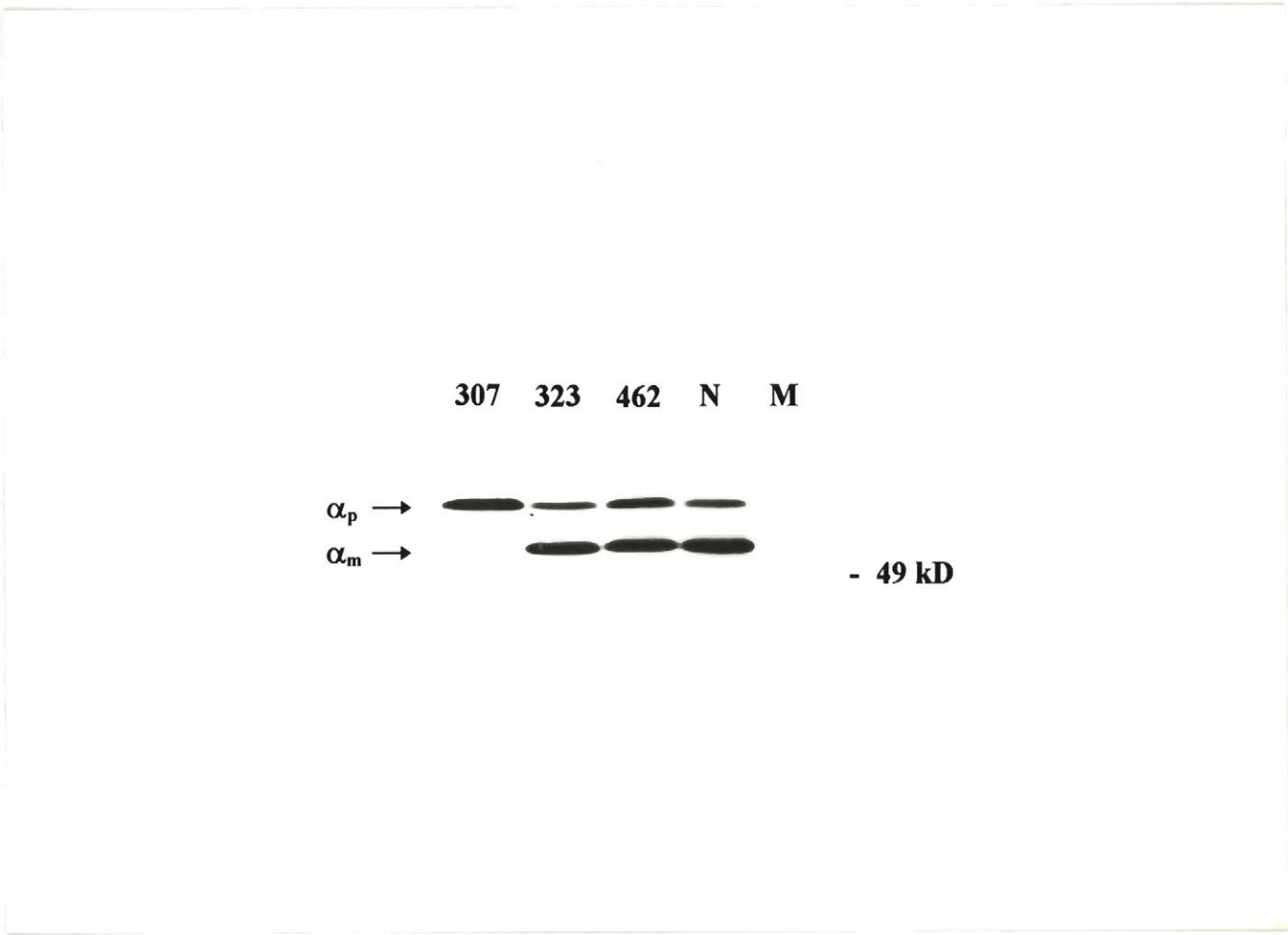

Figure 3. Western blot analysis of NG cells transfected with pCMVE307D, pCMVE323D, pCMVE462D and pCMVα (N). Lane M represents mock transfected cells. The arrows indicate the position of the precursor (α_p) and mature (α_m) α–subunit. The other bands are background bands.

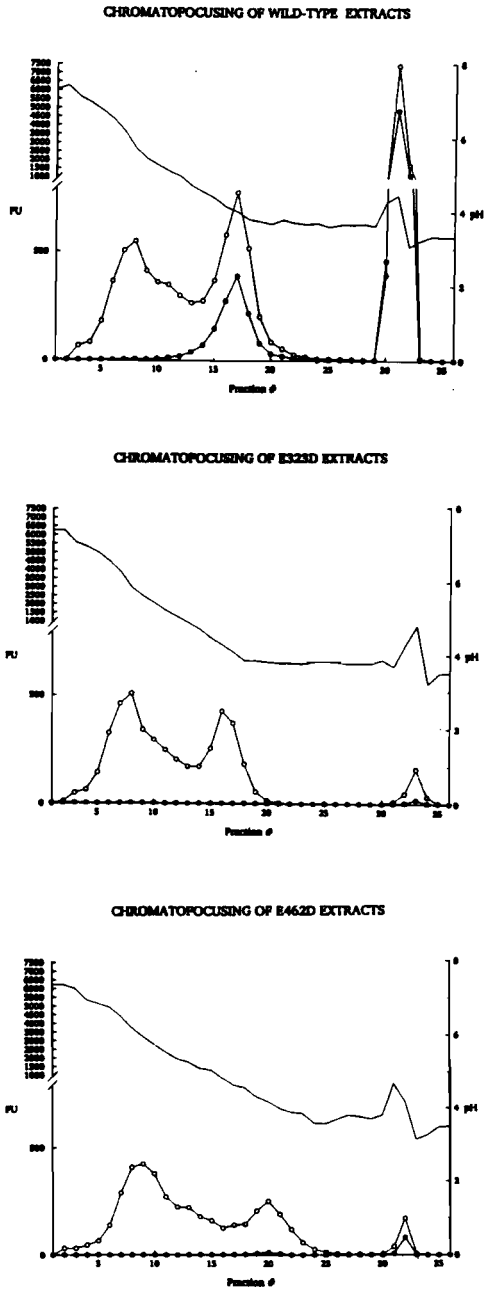

Figure 4. Chromatofocusing of NG extracts transfected with pCMVα, pCMVE323D and pCMVE462D. Hex B elutes first followed by Hex A and Hex S. Fractions eluted from the column were assayed for 4MUG (open circles) and 4MUGS (filled-in circles) activity. The measured fluorometric units and pH (solid line) are plotted versus fraction number.

5.5 DISCUSSION

Transient expression of TSD-associated mutations has previously been reported using COS cells (for review, 16). The principle disadvantage of the COS cell is the relatively high level of endogenous hexosaminidase activity which renders accurate determination of low levels of transfected Hex A activity virtually impossible. Human and Simian hexosaminidases are not usually antigenically distinct. However, an antibody which recognizes only the human β–subunit has been reported (19). Use of this antibody permits accurate assessment of enzymatic activity due solely to the transfected gene when the cells are cotransfected with human *HEXA* and *HEXB* cDNA's. In practice TSD mutations are usually expressed entirely as the non-physiological Hex S isozyme (20, 21, 22) which is both less stable than Hex A and has a different substrate specificity. Levels of Hex S expressed by mutant *HEXA* cDNAs do not usually correlate with residual Hex A activity in patients' cells and therefore do not reflect the wide range of clinical severity associated with the G_{M2} gangliosidoses (16, 17).

Transfection of TSD-NG cells (with the normal *HEXA* cDNA) led to a 400-fold increase in 4MUGS hydrolysis (Table 1). Most transfected normal α–subunit expression occurs in the form of Hex S (Figure 4), most likely due to overexpression of the transfected α–subunit relative to endogenous β-subunits. Nevertheless, significant expression (22%) of the transfected gene product in the form of Hex A occurs as shown by the chromatofocusing profile (Figure 4). The presence of both the precursor (67kDa) and mature (54kDa) forms

(Figure 1, Western analysis) confirmed that the newly synthesized α–subunit is normally processed and targeted to the lysosome. The presence of both forms in cell extracts transfected with the wild-type *HEXA* cDNA contrasts with the absence of the precursor α–subunit in normal fibroblasts. We believe that the high level of expression in NG cells may limit a component of the processing or targeting pathway.

We have analyzed the expression in NG cells of *HEXA* mutations associated with adult-onset, juvenile-onset and infantile-onset TSD. The level of residual enzymatic activity expressed by these transfected mutant cDNA's correlates well with the severity associated with these mutant alleles (phenotype/genotype correlation). In the case of the R170W mutation, these results confirm our previous report (7), that this mutation causes infantile TSD.

We have also applied transient expression in NG cells to the investigation of amino acid residues which have been predicted to function as components of the catalytic site. The active sites of glycosidases are believed to consist of a pair of dicarboxylic acids which fulfill a proton-donating function and a nucleophile role (23). The identity of the catalytic residues in Hex A has been the subject of intense investigation (7,19, 24, 25, 26, 27).

We previously reported the occurrence in a TSD patient of the mutation D258H (7) and demonstrated that this patient had the B1 biochemical phenotype (6). Patients with this phenotype have normal levels of Hex A when assays are performed with the 4MUG

substrate but have no activity when 4MUGS is used. It has been proposed that such mutations affect the catalytic site of the α–subunit (6,24,29). Consistent with such a role for the Asp258 residue is the finding that in fibroblasts of patients with the αD258H allele Hex A has a greatly decreased Vmax for the α–subunit specific substrate and no change in the Km (6).

Three mutations at codon 258 were analyzed. All three mutants, D258H, D258E and D258N, were compatible with the expression of the precursor α–subunit and, with varying degrees, of its conversion to the mature form (Figure 2). Enzymatic activity was reduced, in all cases, to less than 1% of that expressed when NG cells were transfected with normal *HEXA* cDNA (Table 3). Since the isofunctional mutation at this residue not only affects structure but also function, the role of this residue in catalysis, if any, remains unknown. Furthermore, D258 is conserved in all members of family 20 except Porphyromonas gingivalis.

The hexosaminidases were recently assigned to family 20 of glycosyl hydrolase enzymes according to the classification system of Henrissat (8). This system depends more on sequence similarity than does the EC classification system in which enzymes are grouped on the basis of substrate specificity and common reaction mechanism. Members of each homology family generally share the same folding characteristics. Catalytic residues within families are absolutely conserved (9). A combination of hydrophobic cluster analysis and other homology methods (8) were used to develop hypotheses regarding the functional roles

of specific amino acid residues in family 20. In this family, glutamate residues at sites homologous to Hex A α–subunit positions 307, 323 and 462 are conserved in all members. Tews et al (10) reported the first X-ray diffraction structure of a member of family 20; a chitobiase from *Serratia marcescens*. The catalytic residue in this protein corresponds, on the basis of homology, to E323 of the *HEXA* gene product.

The role of the three Hex A glutamate residues (E307, E323 and E462) was analyzed by expression of mutant *HEXA* cDNAs carrying glutamate to aspartate substitutions at each of the positions. Expression of all three mutant cDNAs in TSD-NG cells resulted in reduced 4MUGS hydrolysis activity (Table 4). The E307 residue is unlikely to perform a catalytic function since the conservative E307D mutation interferes with the maturation of the α–subunit (Fig 3).

Our data suggest that residues 323 and 462 may play important catalytic roles in human Hex A. The conservative isofunctional mutations at these sites produce catalytically defective but apparently normally folded α–subunits. E323 is the strongest candidate for a catalytic residue since i) it is absolutely conserved in family 20, ii) the corresponding residue in chitobiase has been identified as a catalytic residue and iii) the E323D mutant is compatible with the synthesis of a Hex A whose α–subunit is catalytically defective toward 4MUG and 4MUGS hydrolysis. E740 in chitobiase, the equivalent of E462 in *HEXA*, is located in the active site but does not seem to play a catalytic role. However, the demonstration that an isofunctional exchange in this residue is compatible with a normally folded Hex A

combined with the evidence that E462 is highly conserved in family 20 suggests that it is a strong active residue candidate. The precise positioning of these carboxylate groups may be critical for catalytic function as is the case for endo-1,3-1,4-β–glucanase of *Bacillus marcerans*. A shortening of the active site residues, E103 and E107, side chains by one methylene group completely inactivates the enzyme (28).

Liessem et al (27) recently reported that E355 in the β–subunit of Hex A, the equivalent of E323 in the α–subunit, is located in the substrate binding site of Hex B. The photoaffinity label, [^{3}H]-1-ATB–GalNAc, was used to identify E355 as a component of the active site. Based on the position of the photoreactive site from the bound sugar in [^{3}H]-1-ATB–GalNAc, the authors suggest that E355 is not involved in glycosyl bond cleavage. Detailed kinetic and crystallographic studies on these and other expressed *HEXA* mutants will be necessary to unambiguously identify all the active site residues in this enzyme and their role in the reaction mechanism.

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CHAPTER 6

DISCUSSION

6.0 DISCUSSION

This thesis encompasses several different aspects of TSD which range from the evaluation and improvement of the heterozygote screening test to structure-function studies on the enzyme hexosaminidase A.

Screening for TSD is a prototype for the prevention of other Mendelian disorders. The quality of this program, its impact on the populations at risk and its evolution under the influence of new technology is, and should be, continuously evaluated. In order to assess the impact of the emerging techniques of molecular biology on carrier screening, we compared the conventional biochemical screening test to the detection of the carrier state using DNA diagnostics.

The identification of new mutations, via the ascertainment of affected individuals led to the characterization of four novel *HEXA* mutant alleles. One of these alleles carries a putative active site mutation (D258H). The functional role of this residue, and three other active site candidate residues identified in structural and homology studies, was investigated in a novel transient expression system. The features of this new expression system are compared to the commonly used expression system, the COS cell.

A splicing mutation causing chronic TSD was also identified. The effect of the mutation on splicing was determined and the residual amounts of normal and misspliced mRNA were quantitated using competitive PCR.

6.1 TSD Screening

Since several variables of the assay conditions can affect measurement of enzymatic activity and thus influence the results of a screening test, it is imperative that appropriate carrier and non-carrier enzyme activity ranges be determined by each screening centre. In particular, it is of utmost importance that each laboratory define its 'gray zone' or that range of enzyme values within which conclusive assignment of genotypes can not be made (Blitzer and McDowell, 1995). The existence of a gray, or overlap, zone between hexosaminidase activity values for heterozygous and homozygous normal individuals is inevitable in any statistical test (Gold et al, 1974). Its consequences are that some individuals will be either unclassified or misclassified as false negatives (normal test results for true heterozygotes) and others as false postives (carrier test results for homozygous normal individuals).

With the advent of molecular diagnosis for genetic disorders it became posssible to employ binary tests to detect with absolute accuracy the presence or absence of a single mutant allele. The new technology thus led to the comparison of the enzyme assay to DNA diagnostic procedures for identification of TSD heterozygotes among the same group of tested individuals. Since slightly different methods are used to differentiate

hexosaminidase isoenzymes in centres across the world and since the normal and carrier ranges vary between centres, it is important that DNA and enzyme tests be compared in each centre. To date however, only a few such studies have been reported (Triggs-Raine et al 1990; Paw et al, 1990; Grebner and Tomczak, 1991; Fernandes et al, 1992a).

In this study, the DNA and enzyme tests were compared on blood samples taken from 78 individuals of Ashkenazi-Jewish origin who participated in heterozygote screening at the Quebec TSD Screening Centre in Montreal (Fernandes, 1992). Individuals scoring as positive with one of the three allele-specific DNA diagnostic tests but in the normal range for serum hexosaminidase A were considered to be "false negative". Those scoring negative on all three DNA diagnostic tests but in the carrier range for serum hexosaminidase A were considered to be "false positives". For genetic counselling purposes, the boundary between carrier and normal values for the serum enzyme assays was initially defined to increase "sensitivity" (the ability of the test to identify those individuals who possess a mutation) at the cost of "specificity" (the ability to exclude from classification as mutants those who have only the wild genotype). The relationship of these two screening parameters to the counselling process is discussed in Scriver and Hechtman (1979). Thus, a percentage of normal individuals will be misclassified (false positives) to increase the sensitivity of the assay. Our data substantially reproduced the predicted level of accuracy of the serum test (Gold, 1974).

In a study such as the present one, it is only the percentage of false negatives which permit one to draw conclusions about the efficiency and accuracy of the biochemical screening program. Thus approximately 1 in 25 individuals, known by DNA diagnostics to be a TSD heterozygote, are not detected in serum screening. As the above discussion suggests, the boundary defining the "gray zone" can be adjusted to include a greater number of low serum Hex A individuals as carriers but only at the expense of increasing the number of false positives. A better solution would be to perform DNA diagnostic tests on samples from individuals whose test value puts them in or close to the "gray zone". The false positive individuals detected in this comparison will be composed of two groups: those who are homozygous for normal genes but who are on the low end of the range of normal serum hexosaminidase A values and those who are truly carriers of a mutation which reduces normal enzyme expression but is not one of the alleles for which the population was tested. Our results, therefore, indicate that at least 96.4% of the Ashkenazi-Jewish carrier population possesses one of the three high frequency mutant alleles known to occur in this population. The remainder of the "carrier" population may have other TSD alleles or pseudodeficiency alleles, or may truly be normal homozygotes with low enzymatic activity. The false positive rate of other TSD screening centres who compared enzymatic results of carrier testing of Ashkenazi-Jewish populations with molecular analysis of the same three mutations was similar to that reported here except for the Triggs-Raine (1990) study. The significantly higher false positive rate (18%) in the latter study may be due to unidentified mutations or alternatively, a "boundary" may

have been used by either of the two participating centres (Toronto and Boston) that is more "sensitive" and less "specific" than that employed by the Montreal centre.

These studies also aimed at determining relative frequencies in the Ashkenazi Jewish population of the three TSD mutant alleles, the pooled results of all studies were: TATC1277, 75 to 80%; IVS12, +1 G->C, 15% and of G805A, 3%. Two percent of TSD carriers in this population are thought to have benign mutations (M.M. Kaback, unpublished observation) resulting in a detection rate for carriers of over 95%.

A general consensus on the use of DNA diagnostics in TSD heterozygote screening is still elusive. For high-risk populations where the mutation spectrum at the *HEXA* gene locus is known (such as the Ashkenazi Jews or the French Canadians) and where a limited number of different allele-specific tests can be employed to ascertain >95% of carriers it would appear that DNA diagnostics could effectively replace serum enzyme assays with equal sensitivity and a higher specificity (i.e. DNA tests should not result in any false positives). However, to the extent that a diagnostic laboratory is required to determine genotypes of individuals who do not belong to these high-risk groups, the sensitivity of testing would decrease if DNA diagnosis were to replace serum enzyme assays. Therefore, I favor the use of the enzyme assay as the primary screening test.

The recent identification of two pseudodeficiency alleles (Triggs-Raine et al, 1992; Cao et al, 1993), and possibly a third (R249Q, Callahan et al, 1995), at the *HEXA* locus has

emphasized the importance of DNA diagnostics in TSD screening (Trigss-Raine et al, 1992; Cao et al, 1993). A high frequency (35%) of these mutations is found in non-Jewish TSD heterozygotes ascertained by serum hexosaminidase assays. Therefore, enzymatically-identified carriers who are not from an Ashkenazi-Jewish background should also be DNA-tested for the presence of pseudodeficient alleles. The DNA test should also be used to confirm all borderline results. Leukocyte testing should be used only when testing of a serum sample persistently gives ambiguous results.

In considering arguments for enzyme versus DNA testing the monetary cost of both procedures is certainly of some importance. The cost of DNA testing is presently $150 to $200 per person. This should decline with the rapid developments in technology and automation (Kaback, 1993). However, for the present, it is simply not cost-effective to completely replace serum enzyme assays with DNA diagnostics even in populations whose alleles have been well characterized.

Heterozygote screening identifies couples at risk and thus requires programs for prenatal diagnosis and it is these types of programs that are most likely to evolve in the immediate future.

6.2 Four novel TSD alleles

It was initially assumed that all infantile TSD patients harbored the same *HEXA* mutation (O'Brien, 1983). However, since the application of polymerase chain reaction technology to the characterization of human mutations, it is now understood that allelic heterogeneity occurs in both population groups that are at high risk for this disorder and that among patients from other ethnic groups.

In this study, I have contributed to the characterization of the G_{M2} gangliosidosis mutation spectrum. The four mutations identified were shown to be disease related on the basis of : (i) the presence of the putative mutation in the patient as shown by allele-specific oligonucleotide analysis or restriction digestion (sequencing analysis does not suffice), (ii) the presence of the mutation in family members when DNA was available and (iii) expression analysis of *HEXA* cDNA carrying the mutation (not absolutely necessary for null alleles).

The four mutations were identified in three patients. Two of these were infantile patients with the B1 phenotype (Fernandes et al, 1992b) and the third had the chronic form of G_{M2} gangliosidosis (manuscript in preparation). The two B1 patients were compound heterozygotes for the D258H allele and a second allele, R170W or del CT927-928. Since the latter allele is a null mutation, the B1 phenotype was assigned to the D258H allele.

Biochemical analysis of residual Hex A in the patient with the D258H /del CT 927-928 genotype revealed a drastically reduced Vmax, an unchanged Km for 4MUGS and a downward shift of the pH optimum (Bayleran, 1987). On the basis of these observations it was suggested that D258 was an active site residue. Expression analysis in NG cells confirmed that this allele is responsible for the TSD phenotype since cells transfected with the αD258H cDNA exhibit no significant cleavage of 4MUGS. When transfected cells were analyzed by Western blot it was apparent that the D258H mutation affects not only catalytic activity but also the rate of maturation of the pro-α-subunit to the lysosomal (54 kD) form of the enzyme. Since it was not clear whether the effect on catalysis and the effect on processing were related we analyzed alleles carrying either a isofunctional (D258E) or an isosteric (D258N) substitution at this codon. The former mutation resembles the patients' allele in that both reduction of processing and loss of catalytic activity occur as a consequence of the same mutation. However, the D258N allele produces an α-subunit which is converted to the 54 kD form at least as effectively as the wild-type α-subunit but has < 0.5 % of wild-type catalytic function.

These observations uphold the hypothesis that D258 is required for catalytic function, however they do not address the question of whether D258 participates in catalysis (i.e. by donating protons or stabilizing the transition state) nor do they demonstrate whether this residue is physically present at or near the catalytic site.

Another B1 mutation in the *HEXA* gene, R178H, has been analysed by Brown and Mahuran (1991). Expression in COS cells of a conservative substitution at the homologous position in the *HEXB* gene, R211K, produces a species of Hex B that has no catalytic activity but which appears to undergo normal folding, dimerization and to bind substrate with a Km comparable to that of the wild-type enzyme. Although it has been suggested that R178 is involved in the active site of the α-subunit, this notion is difficult to reconcile with the proposed acid-catalyis reaction mechanism common to glycosyl hydrolases. It may play a significant role in the active site but it's unlikely to be directly involved in catalysis. Brown and Mahuran (1991) have proposed that R178 participates in a "charge-relay network" in Hex A.

The expression of αR170W cDNA in NG cells confirmed it as a disease-causing allele. The role of this residue, however, in the protein is still unknown. A second mutation, R170N (Nakano et al, 1990), has been reported at the same codon, an apparent CpG hotspot for mutations. The R170N patient does not have the B1 phenotype but produces an unstable mutant α-subunit which does not exit from the RER. The stability of Hex A carrying the R170W substitution was not tested but like R170N mutant enzyme, it does not mature. Mutations at this residue may affect the glycosylation of the protein since it is located 13 amino acids away from one of the three glycosylation sites and thus substitution may hamper subsequent processing and targeting events that depend upon glycosylation (Nakano et al, 1990).

The R170W and D258H mutations are interesting from the perspective of population genetics. The R170W mutation was identified in a unrelated patient living in the United States, whose ancestral origins trace back to the Estrie region of Quebec.

The centre of diffusion of the most frequent allele in the French-Canadian population (7.6 kb deletion at the 5' end of the gene) is the Gaspe region of Quebec (de Braekeleer et al, 1992) and for the intron 7, +1 mutation the Saguenay-Lac-St Jean region (Hechtman et al, 1992). The R170W mutation however, may represent a third TSD allele unique to the French-Canadian population and arising from yet a third centre of diffusion for TSD alleles in Quebec. A second, unrelated individual from the Estrie region with the R170W mutation has already been identified supporting this hypothesis. Two other individuals from two independant studies were found to carry this allele suggesting that this variant allele is not 'private' (Triggs-Raine et al, 1995).

The D258H allele in the two patients studied, as well as in their parents, also carried the *HEXA* polymorphism IVS13, -6 T->C (Kaplan et al, 1993). Both unrelated families were Americans of Scots-Irish descent. A third TSD patient carrying the D258H mutation has been reported (Cotton, 1993). It would be of interest to test this third family for the IVS13 polymorphism.

The ethnogeographic distribution of this polymorphism is unusual. It occurs on 1.4% of normal chromosomes in Ashkenazi Jews who have ancestry in Lithuania, Belarus and Ukraine. It has not been detected in non-Jewish Lithuanians, Jews of Sephardic origin or

in several other ethnic groups. Therefore, the ethnic origin of the IVS 13 chromosome associated with the G772C mutation does not seem to be the same as that of the normal IVS13 carrying chromosome. Since this polymorphism does not involve a highly mutable CpG site and occurs in two unrelated populations, it is probably one of the more ancient TSD mutations.

The IVS7, -7 G->A mutation was identified in a chronic TSD patient (manuscript to be submitted). Mutations occuring at or near the 3' splice site (3'ss) may either result in i) the use of the acceptor site of the next intron downstream (skipping the intervening exon), ii) the utilisation of a cryptic splice site and iii) reduction of the amounts of RNA. The majority of 3' ss mutations are located at the last two invariant nucleotides of the intron (Krawczak et al, 1992). The IVS7, -7, G->A was considered to be of interest since the characterization of its effects on mRNA expression could contribute to defining the boundaries for the requirements of the splice consensus region. Furthermore, one of the consequences of the IVS7, -7 G->A mutation is that it creates a new 3' splice site which may or may not be used by the splicing machinery of the cell.

I observed that the IVS7, -7 G->A mutation has two effects on mRNA splicing. The primary effect is to greatly reduce the amount of mRNA expressed and the second effect is that a portion of this mRNA has a deletion of exon 8. Since the intact deletion of exon 8 is out of frame, the protein made (if any) is presumably inactive.

The mechanism governing splice site selection is still an enigma. It not only seems to depend on the splice site consensus sequences but also the surrounding primary sequence and RNA secondary structure (Menichini et al, 1994). According to the 'exon definition' model proposed by Robberson et al (1990), the spliceosome complex (U2 and U5 snRNPs) initially interacts with the 3' acceptor site and then searches for a suitable 5' splice site (ss). Once the 5' ss is recognized, U1 and associated proteins bind and the exon is considered an individual unit by other splicing factors that subsequently interact with the 5' and 3' ss. This model could explain the effect of the IVS 7 mutation on splicing. The mutation at the -7 position could affect U2 binding and result in the search for another acceptor site, in this case, that of intron 8. We did not detect any mRNA splice products that used the AG site created by the mutation. Competitive PCR revealed that both the normal and exon 8 deleted species are present in equivalent amounts.

Three other adult TSD mutations have been reported, namely G269S, K197T and possibly Y180H. The G269S occurs in approximately 3% of Ashkenazi Jewish carriers whereas the other two and the IVS7 mutation seem to be 'private'.

Considerable clinical variability has been observed in patients, even between siblings, who are either homozygous for the G269S mutation or are compound heterozygotes for this allele and for a null TSD allele (Navon et al, 1986; Federico et al, 1991). The reasons for these variable phenotypes are not known. Since the mutation G805A occurs at the last base pair of exon 7, it has been proposed that alternative splicing could be

involved in producing different amounts of normal mRNA in different patients carrying the G269S mutation but having different neurological phenotypes. Such a mechanism was proposed by McInnes et al (1992) for the P417K SD mutation. However, RTPCR of mRNA from patients' fibroblasts revealed no alternatively spliced products and a normal amount of G269S message.

6.3 Expression studies

The most commonly used system for the expression of *HEXA* and *B* mutant alleles is the COS cell. The major disadvantage of this transient expression system is the presence of endogenous (simian) hexosaminidases which are not readily distinguishable from the expressed human enzymes. In order to overcome the difficulties associated with the COS system I have established a novel transient expression system for the study of *HEXA* mutant alleles which uses cells that have very low levels of 4MUGS hydrolysis. The novel system, which employs SV40 transformed neuroglial (NG) cells obtained from an infantile TSD patient, permits the study of low activity *HEXA* mutants and has also proven useful for Hex A structure-function studies (manuscript to be submitted).

NG cells transfected with the wild-type *HEXA*-cDNA (pCMVα) exhibit on average 400-fold higher rates of cleavage of 4MUGS relative to mock transfected control cells. In wild type transfectants both Hex A and Hex S are produced. By contrast, expression of wild type *HEXA* cDNA by transfection of pSVLα into COS cells generally increases 4MUGS hydrolysis approximately 3-fold compared to mock transfectants and, in the case

of the COS transfectants, all of the expressed activity is in the form of Hex S (Brown and Mahuran, 1993). This dramatic difference in the increase in 4MUGS hydrolysis has two components. In the first instance levels of endogenous α-subunit in the NG cell are much lower than in the COS cells and secondly the CMV promoter is much stronger than the SV40 promoter present in the pSVL vector. Table 1, in chapter 5, indicates that both factors contribute to the greater relative *HEXA* cDNA expression in the NG system (compare pSVLα to pCMVα at 48 hours).

The NG transient expression system was used to assess genotype-phenotype correlations for *HEXA* mutations associated with different levels of clinical severity. As expected, expression of the infantile TSD allele, R170W, produced no 4MUGS hydrolysis. However, the percent residual 4MUGS hydrolysis, relative to enymatic activity of wild type *HEXA* transfectants, for the G269S (adult-onset TSD) and G250D (juvenile-onset TSD) mutations was similar to that in patients' fibroblasts. The expression of these three mutations in COS cells results in no significant difference between infantile-onset and juvenile-onset TSD mutations (unless these mutant genes are cotransfected with plasmids containing the human *HEXB* cDNA) (Brown and Mahuran, 1993). Expression of the αG269S mutation in COS yields inappropriately high levels of Hex S. Thus, the results of expression of clinically significant mutations indicate that it should be possible to use the NG expression system to make predictions about phenotypes associated with new *HEXA* mutations. Such analyses might be particularly important for mutations identified in clinically normal heterozygous individuals or compound heterozygotes.

Western blot analysis confirmed that the increase in 4MUGS hydrolysis post-transfection was due to the presence of newly synthesized α-polypeptide. The presence of both the precursor (67 kDa) and the mature (54 kDa) forms confirms that a proportion of the newly synthesized α-precursors are being targeted to the lysosome. This finding is strikingly different from results of western analysis performed on normal human cultured fibroblasts where the relative amount of precursor α-subunit is so small that it can be detected only by using pulse-metabolic labeling. Pulse-labelling experiments in cultured human fibroblasts obtained from patients with SD revealed that a small proportion of precursor α–chains are converted to the mature form (Proia et al, 1984). It is likely that in the NG transient expression system the targeting mechanism is overwhelmed by expression of large amounts of α-subunit and that some component of the targeting pathway becomes limiting.

The large amounts of Hex S present in the wild-type transfection chromatofocusing profile is a result of excess expression of α-subunits relative to β-subunits. The presence of Hex B in chromatofocusing profiles of NG cells transfected with wild type *HEXA* cDNA despite the overwhelming expression of α-subunits is explained by the limited transfection efficiency which varies between 10 to 20%. That is, Hex B formed represents the contribution of the 80-90% of cells which are untransfected.

6.4 Active-site studies

Hexosaminidases, like other glycohydrolases, cleave their substrates via an acid-catalysis mechanism (Sinnott, 1990). This reaction mechanism requires a proton donor and a nucleophile/base, neither of which have been identified in human hexosaminidases. For glycohydrolases for which three dimensional structures have been determined, aspartic acid, glutamic acid and tyrosine residues have been identified as proton donors and aspartic acid and glutamic acid residues as the base/nucleophile (Svensson and Sogaard, 1993).

Homology modelling permits drawing of inferences from a known three dimensional protein structure to related (uncrystallized) proteins provided all the proteins compared are related to each other by sufficient primary sequence homology. Certain inferences regarding the active site residues of human Hex A were made using a combination of homology analysis of enzymes belonging to glycohydrolase family 20 and comparison of conserved residues in this family to tertiary structure data obtained from the recently crystallized Chitobiase from *Serratia marcescens* (CHS).

Three putative active site residues in the Hex A α-subunit (E307, E323, E462) were identified on the basis of their absolute conservation in members of glycohydrolase family 20. Expression analysis of mutant α-cDNAs carrying E307D, E323D and E462D substitutions respectively suggested that E323 and E462 may function as active site residues. The mutant α-polypeptides are synthesized, processed and targeted in the same

manner as the wild-type α-chain, as determined by Western analysis, but the mutant Hex A's are greatly reduced in catalytic activity. The dramatic effect on enzyme activity as a consequence of a conservative substitution is a finding compatible with an important functional role for the wild-type residue. It has similiarly been shown that shortening, by one methylene group, of the side-chain of the active site residues E103 and E107 of *Bacillus marcescens* endo-1,3-1,4-β-glucanase completely inactivates the enzyme (Hahn et al, 1995). The precise positioning of these carboxylate groups in the active site of hexosaminidases may be critical for their catalytic function. Presumably an isofunctional mutation changes the spatial position of the carboxylate group while preserving its electrical and hydrophilic character as well as the chemical properties of other residues composing the catalytic cleft (Knowles, 1987). However, isofunctional mutations may affect the structure of a protein either globally or locally. Western analysis and chromatofocusing experiments and measurement of β-subunit catalytic function in mutant Hex A's confirmed that the E323D and E462D mutants did not undergo any major structural change as a result of the mutation.

The presence in lysates of cells transfected with these mutant cDNAs of precursor and mature α-subunits in quantities similar to those of the wild-type transfectants indicate that there were no global effects on the mutant polypeptides resulting in either loss of precursor in the RER or failure to dimerize with β-subunits. The mutant α-chains were appropriately processed and targeted to the lysosome. The mutant Hex A had a pI similar

to that of the wild-type enzyme and is able to hydrolyze 4MUG due to the presence of the β-subunit, but is not able to hydrolyze 4MUGS. These two mutants may be thought of as the first "human-made" B1 mutations. By contrast the E307D mutation produced significantly reduced amounts of mature α-subunit suggesting that the E307 residue may not play a catalytic role in Hex A.

The X-ray crystallographic map of CHS indicates that the residues corresponding to E323 (CHS539) and E462 (CHS740) are located in the active site of the enzyme, in the β4 and β8 loops, and that CHS539 is a catalytic residue. By extrapolation E323 is an active residue in the human α-subunit. Residue E355 in the β-subunit, which is homologous to αE323 has recently been shown to be a component of the Hex B active site using photoaffinity labeling (Liessem et al, 1995). Incubation of Hex B with the photoaffinity label, [^{3}H]-1-ATB-GalNAc, yielded one prominently labelled peptide composed of amino acids 339 to 359. The photoaffinity label was bound exclusively to E355. The authors suggest that this residue is located at the substrate binding site of the β-polypeptide.

The role of E462 in catalytic function is less obvious. Even though its CHS homolog, E740, is also located at the active site its role has yet to be determined.

Although these results clearly show that E323 and E462 are strong candidates for catalytic residues of the α-polypeptide, further studies including site-directed mutagenesis (isosteric mutations and double mutants), kinetic analysis and x-ray crystallographic

analysis of substrate-bound hexosaminidase are necessary to provide further evidence for the participation of these residues in the catalytic mechanism of hexosaminidases. Kinetic parameters such as Km, Vmax, pH optima, and thermostability of the two mutant enzymes will offer more insight into the roles of E323 and E462. If these residues are, in fact, components of the Hex A active site then one would predict that mutant Hex A's with conservative substitutions at these sites would have drastically reduced Vmax but normal Km, provided, of course that when mutant α-cDNAs are expressed there is sufficient residual Hex A activity for this kind of kinetic analysis.

Residues other than the catalytic amino acids (the acid and base) have been shown to be involved in glycosidic bond cleavage. Three arginines, a glutamic acid and a tyrosine, are found in the active site of a bacterial sialidase (from *Salmonella typhimurium* LT2). The arginine residues interact with the carboxylic acid group of the sialic acid moiety. The glutamic acid residue stabilizes one of the triad arginines and the tyrosine residue stabilizes the transition state. (Crennell et al, 1993).

Homology analysis of family 20 glycohydrolases also revealed four absolutely conserved aspartic acid residues. Since the catalytic residues within a family are absolutely conserved and the active residues in CHS are glutamic acids, it is unlikely that these aspartic acids are directly involved in catalysis.

To date, 22 of the 52 glycohydrolase families presently recognized by the Henrissat classification system have at least one member crystallized. Comparison of the 3 dimensional structures of these crystallized enzymes suggests that tertiary structure is more conserved than primary amino acid sequence. The tertiary structural similarity between families 1, 2, 5, 10, 17, 30, 35, 39 and 42 suggests that these proteins evolved from a common ancestor (Henrissat et al , 1995; Jenkins et al, 1995). An evolutionary relationship of this kind has not yet been identified for family 20.

The overall topology of the active sites of glycosyl hydrolases fall into three classes, regardless of whether the catalytic event retains or inverts the anomeric carbon (Davies et al, 1995). The topologies are refered to as; pocket/crater, cleft/groove or tunnel site. Hexosaminidase is likely to have pocket topology since this type of site is considered optimal for the action of exohydrolases.

The three dimensional structure of human hexosaminidase will no doubt be determined in the near future and this will offer a deeper understanding of the mode of function of this clinically important protein.

CHAPTER 7

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REFERENCES

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APPENDIX I

A *PST*+ POLYMORPHISM IN THE HEXA GENE WITH AN UNUSUAL GEOGRAPHIC DISTRIBUTION

A *Pst*+ Polymorphism in the HEXA Gene with an Unusual Geographic Distribution

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ABSTRACT

A polymorphic variant in the human HEXA gene is described. This gene encodes the α-subunit of hexosaminidase A, the enzyme which is deficient in Tay-Sachs Disease (TSD). In individuals carrying the polymorphism there is a T->C transition at position -6 in intron 13. The substitution creates a site for the restriction endonuclease *Pst1*.

This variant has an unusal ethno-geographic distribution. It occurs on 1.4% of non-TSD carrier chromosomes in Ashkenazi-Jews. All individuals ascertained carrying the *Pst+* allele have ancestry in Lithuania, Belarus and Ukraine. By contrast, no individuals carrying the *Pst+* allele have been detected among non-Jewish Lithuanians, Jews of Sephardic origin or in several other ethnic groups. Two unrelated non-Jewish families have been identified in which the *Pst+* variant occurs. In both cases the variant occurs on a chromosome carrying a novel TSD mutation (G772C) associated with the B1 phenotype. The *Pst+* G772C chromosomes are of Scots-Irish descent.

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INTRODUCTION

The HEXA gene, in which more than 30 mutations occur leading to variant forms of the neurodegenerative disorder Tay-Sachs disease (TSD), spans 35 kb on chromosome 15q22-24. TSD has elevated frequency in Ashkenazi Jews where 3 mutations predominate; a 4 bp insertion in exon 11 (73% of carrier chromosomes), a splice junction mutation in intron 12 (14%) and a G805A substitution in exon 7 (3%) (1-3). The historical center of diffusion for the exon 11 allele is believed to be in central Europe (corresponding to Austria, Hungary and Czeckoslovakia) (4).

We describe a polymorphism in the HEXA gene creating a new site for the restriction enzyme *Pst*1 with an allele frequency of 1.4% in Ashkenazi Jews. Individuals carrying this Pst^+ allele trace ancestry to Lithuania and Belarus suggesting a center of diffusion different from that of the most common TSD gene. The Pst^+ allele was also found in two unrelated non-Jewish TSD patientsin whom it occurred in association with a novel TSD mutation (G772C) with B1 phenotype (5,6). In both families the carriers of this chromosome were of Scots-Irish descent.

MATERIALS AND METHODS

Subjects DNA samples were obtained from Ashkenazi Jewish participants in the Montreal (N=97) and California (N=65) TSD screening programs, and from Israel (N=39) and Lithuania (N=2). Sephardic Jewish (N=9) and Italian (N=76) samples were obtained from the Montreal ß-thalassemia screening program. French Canadians (N=36) and French (N=50) individuals were participants in a research program analyzing population origins. Samples from individuals of Scots-English-Irish descent were from Montreal (N=154). California (N=43) and from the United Kingdom (N=6). Samples obtained from individuals of eastern European origin were provided either by the California TSD screening program or were from Lithuania (N=12).

Patients carrying a novel TSD gene (G772C; B1 phenotype) were previously reported (5,6).

Determination of TSD Carrier Status Samples from Montreal, California and England were ascertained for carrier status as previously reported (7,8). Samples from Israel were screened for the 3 common Ashkenazi-TSD alleles.

Identification of *Pst*$^+$ Polymorphism DNA was isolated from blood leukocytes or fibroblasts by standard procedures. Primer pairs used to amplify exon 14 and flanking sequences of HEXA gene, including intron 13, were as reported (9). Single stranded

conformational polymorphism (SSCP) analysis was performed according to Orita et al (10) as modified by Triggs-Raine (9). Direct sequencing of the DNA fragment was carried out using a sequenase kit according to Wong et al (11). Following *Pst*1 digestion of the amplified DNA, samples were electrophoresed on 1.4% agarose.

RESULTS AND DISCUSSION

SSCP analysis of the HEXA gene fragment containing the *Pst*$^+$ allele yielded an altered electrophoretic pattern (Fig. 1). Direct sequencing of the PCR-amplified fragment identified a T->C transition at position -6, in intron 13 (Fig. 2). This mutation creates a *Pst*1 site (Fig. 3). A screen of 203 Ashkenazi Jewish individuals (160 normal, 43 TSD heterozygotes) identified 5 *Pst*$^+$ alleles (frequency = 1.4% on normal chromosomes). No *Pst*$^+$ alleles were observed on TSD chromosomes (Table 1). Three of the five *Pst*+ individuals came from the Montreal sample, one from the Israel group and one from the California sample. A survey of normal individuals of varied ethnic background did not identify any more *Pst*$^+$ alleles (Table 1).

The *Pst*$^+$ variant was also found in 2 patients on chromosomes carrying a novel G772C TSD allele. The *Pst*$^+$ allele was confirmed in one of these patients and in his father by direct sequencing (Fig.2).

Since the original description of HEXA gene structure (13), several reports of rare silent mutations have appeared (14-17). To date, however no neutral (benign) polymorphisms (population allele freqency > 1%) have been reported. Although the elaboration of haplotypes at the HEXA locus would facilitate study of the spread of TSD genes and the migration of human populations, HEXA (unlike HEXB) has thus far proved to be quite

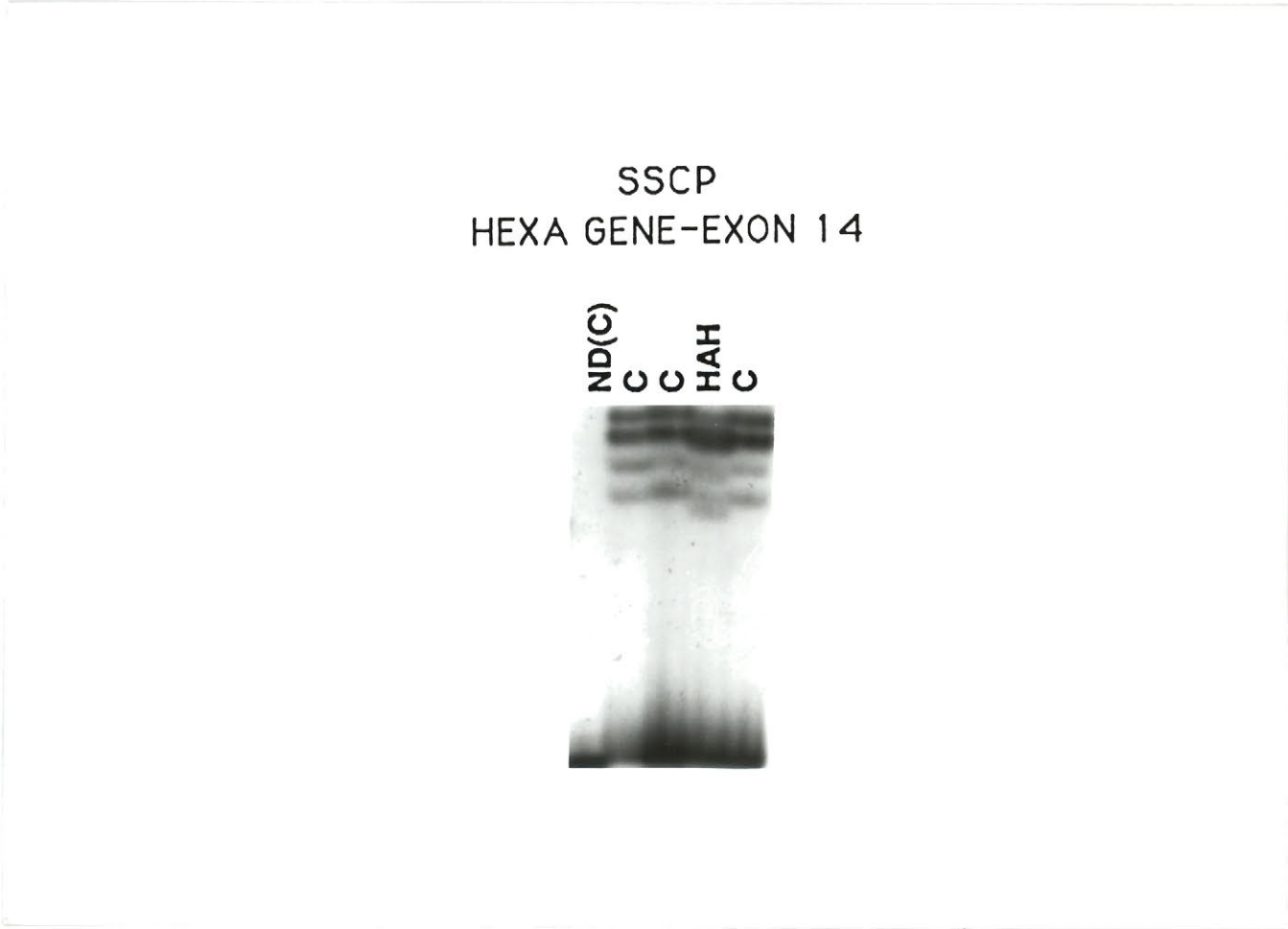

Figure 1 Single stranded conformational polymorphism analysis of HEXA exon 14 and flanking sequences. ND(C) refers to a non-denatured control sample. The three lanes indentified as C are three different control individuals in whom the *Pst*+ variant is not found. HAH is an individual in which the aberrant electrophoretic band is produced by the *Pst*+ variant.

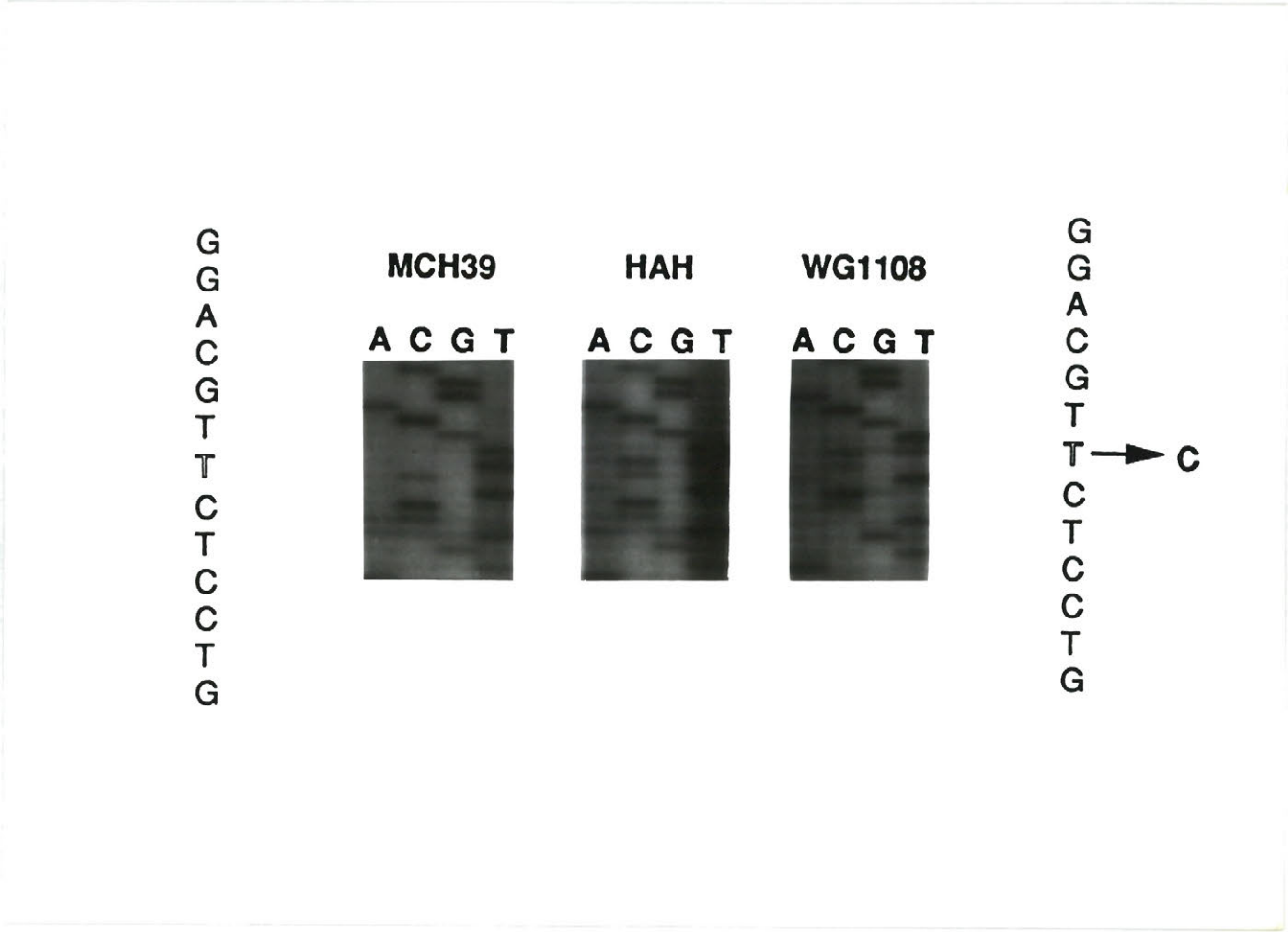

Figure 2 Direct DNA sequencing of amplified HEXA exon 14 products Templates are prepared from control fibroblasts (MCH39), a *Pst*+ individual (HAH), and a TSD patient who is a compound heterozygote for the G772C mutation (WG1108).

Figure 3 *Pst*+ digestion of amplified (HEXA) exon 14 products. Genomic DNA was amplified using exon 14 primers (5'TGACTGGTGTGAAAAGTGTTGC3', 5'CCTTTCTCTCCAAGCACAGG3') and digested with *Pst*1 according to supplier. Phi X174 Hae III digest markers are shown. Lanes 1, 3, 5, 7; amplified DNA. Lanes 2, 4, 6, 8 corresponding *Pst*1 digests illustrating *Pst*+ polymorphisms in 2 Ashkenazi individuals (lanes 4 and 6) and the individual carrying the G772C mutation (lane 8).

Table 1

Chromosomes	*Pst*$^+$	Ethnicity
363	5	Ashkenazi Jewish
43	0	Ashkenazi Jewish TSD
18	0	Sephardic Jewish
384	0	Scots-Irish, English
22	2	Scots-Irish, English TSD
152	0	Italian
100	0	French
72	0	French Canadian
56	0	mixed (unknown)
24	0	Eastern Europe non-Jewish

quite 'barren' of polymorphisms. Such findings are generally rare but have been noted at some other loci of medical interest (e.g. Factor IX) (18).

The *Pst*$^+$ mutation occurs in the pyrimidine tract of intron 13 which is believed to have functional significance. Intronic mutations in the -6 to -10 position are associated with splicing abnormalities leading to disease phenotypes in the ß-globin and the HEXA genes, respectively (9,19). We have no evidence for reduced splicing or altered enzyme activity in *Pst*$^+$ individuals, but believe it to be unlikely since this polymorphism was ascertained predominantly in individuals with normal levels of serum hexosaminidase A.

The Jewish individuals harbouring the *Pst*$^+$ allele each have ancestry in Lithuania and its surrounding regions. The identification of *Pst*$^+$ Ashkenazi-Jewish individuals in samples from three centers (Montreal, California, Israel) makes it unlikely that this variant is "private" in the sense of being confined to a single family. While the *Pst*$^+$ individuals all shared ancestry in and around Lithuania, the Ashkenazi cohort (N=203) represented individuals with ancestry spread over the European continent (Russia, Poland, Germany, etc.) and included relatively few of Lithuanian origin.

The ethnic origin of the *Pst*$^+$ chromosome associated with the G772C (TSD mutation) does not appear to be the same as that of the normal chromosome carrying the *Pst*+ variant. The unrelated carriers of *Pst*+ and G772C were both Americans of Scots-Irish descent. We

therefore surveyed individuals of Celtic (Scottish and Irish) origins (N=203) currently living in England, Ireland and Montreal including 20 who tested postively for the most common 'Irish' TSD gene (IVS 9 splice juntion mutation)(12). No '*Pst*$^{+}$' individuals were identified in this group (Table 1). It is therefore likely that '*Pst*$^{+}$' is in linkage disequilibrium with G772C in this population. The *Pst+* allele, occuring in two unrelated populations and not involving the highly mutagenic CpG dinucleotide, may be more ancient than the common Ashkenazi TSD 4bp insertion-mutation.

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APPENDIX II

CLAIMS TO ORIGINALITY AND CONTRIBUTION TO MANUSCRIPTS

APPENDIX II

Claims to Originality

(i) I assessed the use of DNA diagnostics for the testing of heterozygotes ascertained by the Quebec TSD carrier screening program. This work has been published as Specificity and sensitivity of hexosaminidase assays and DNA analysis for the detection of Tay-Sachs disease carriers among Ashkenazic Jews in Genetic Epidemiolgy 9:169-175, 1992. Although this publication was not the first to consider the use of DNA diagnostics in TSD carrier screening, it was one of a group of studies attempting to determine what adjustments should be made to TSD screening in the age of molecular biology.

(ii) I characterized four novel TSD alleles (R170W, del CT at positions 927-928 or 929-930, D258H and IVS 7, -7 G->A). This represents entirely original work as these mutations have not been detected in other laboratories previous to my studies. The identification of the first three of these mutations was reported in a published article entitled A new Tay-Sachs disease B1 allele in exon 7 in two compound heterozygotes each with a second novel mutation in Human Molecular Genetics 1:759-761, 1992. The fourth mutation, which was further analyzed by competitive PCR, is reported in a manuscript to be submitted entitled A chronic GM_2 gangliosidosis variant with a HEXA splicing defect: Quantitation of HEXA mRNA's in normal and mutant fibroblasts.

(iii) I established a novel and improved expression system for the study of HEXA mutations in an SV40-transformed neuroglial cell line. The cell line was originally described elsewhere but I constructed the plasmids containing the normal and mutant forms of the HEXA cDNAs to be tested, performed the transfections and analysed the expression of the HEXA genes. This work is reported in a manuscript to be submitted entitled: A transient expression system for Tay-Sachs disease genes in cultured neuroglial cells: Expression of active site mutations.

(iv) I investigated the expression of Hex A's carrying mutations at putative active site residues D258, E307, E323 and E462. This work, which is also reported in the above manuscript, complements evidence produced in other laboratories which also points to E323 as an active site amino acid. To my knowledge, none of the other candidates have been investigated for such a proposed role.

(v) I played a secondary role in an investigation that showed linkage disequilibrium between the D258H mutation and the polymorphism, IVS -6, T ->C. This work was published in an article entitled A *Pst*+ polymorphism in the HEXA gene with an unusal geographic distribution in the European Journal of Human Genetics 1:301-305, 1993.

Contribution to each manuscript:

Chapter 2: Genetic Epidemiology 9:169, 1992:

I reviewed the screening files of the past decade and identified potential participants for our study. I was also involved in obtaining the blood samples from each of the individuals and did all the DNA analysis. The manuscript was written by myself, Dr Scriver and Dr Kaplan.

Chapter 3: Human Molecular Genetics: 1:759, 1992:

I did all of the experimental work and the manuscript was written by myself and Dr Hechtman.

Chapter 4: To be submitted:

In this study, I characterized the chronic TSD mutation, designed the standards for competitive PCR and supervised their construction. The actual construction of RNA standards and the quantitation of HEXA mRNA species was performed by Bernard Boulay. The manuscript was written by myself and Dr Hechtman.

Chapter 5: To be submitted:

I did all the experimental work except the experiment in table 1 that was done by Dr Leclerc. In collaboration with Dr Henrissat and Dr Vorgias they gave me access to their homology data that proposed E307, E323 and E462 as candidate active site residues. The manusript was written by myself, Dr Hechtman and Dr Kaplan.

Appendix I:

My contribution to this manuscript was finding the association between D258H and IVS 13, -6 G->C through SSCP analysis of all the HEXA exons of the two patients carrying the D258H mutation.