# CHARACTERIZATION OF THE BENIGN MUTATIONS, R247W AND R249W, ASSOCIATED WITH ß-HEXOSAMINIDASE A PSEUDODEFICIENCY

BY

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A Thesis Submitted to the Faculty of Graduate Studies

in Partial Fulfilment of the Requirements

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## ABSTRACT

Deficient activity of ß-hexosaminidase A (Hex A), resulting from mutations in the *HEXA* gene, typically causes Tay-Sachs disease. However, healthy individuals lacking Hex A activity against synthetic substrates (i.e. individuals who are pseudodeficient) have been described. Most of these individuals have a C739T (R247W) mutation in compound heterozygosity with a Tay-Sachs disease-causing allele. We identified a second benign mutation, C745T (R249W) in a pseudodeficient subject and showed that it accounted for approximately 6% (4/63) of enzyme-defined non-Jewish Tay-Sachs disease carriers. Taken together, the two benign mutations accounted for approximately 38% of non-Jewish enzyme-defined carriers, making their identification an important component of Tay-Sachs disease prevention programs.

To confirm the relationship between benign mutations and Hex A pseudodeficiency and to determine how the benign mutations reduce Hex A activity, each of the benign mutations and other mutations associated with infantile, juvenile, and adult-onset forms of Gm2 gangliosidosis were transiently expressed as Hex S (αα) and Hex A (αß) in Cos-7 cells. The benign mutations decreased the expressed Hex A and Hex S activities toward the synthetic substrate 4-methylumbelliferyl-6-sulfo-ß-N-acetylglucosaminide (4-MUGS) by 60 to 80%, indicating they are the primary cause of Hex A pseudodeficiency. Western blot analysis showed that the benign mutations reduced enzymatic activity by reducing the α-subunit protein level. No change in Hex A heat sensitivity, catalytic activity, or specificity toward 4-MUG and 4-MUGS, were detected in the studies. The effects of benign mutations on Hex A were further analysed in fibroblasts, and during transient expression, using pulse-chase metabolic labelling. These studies showed that (1)

the benign mutations reduced the α-subunit protein by affecting its stability *in vivo*, not by affecting the processing of the α-subunit, ie. phosphorylation, targeting, or secretion, and (2) these benign mutations could be readily differentiated from disease-causing mutations using a transient expression system.

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| | |
|---|---|
| ATP | Adenosine 5′-triphosphate |
| bp | Base pair |
| BSA | Bovine serum albumin |
| cDNA | Complementary DNA |
| dATP | 2′-Deoxyadenosine 5′-triphosphate |
| dCTP | 2′-Deoxycytidine 5′-triphosphate |
| DEAE | Diethylaminoethyl |
| ECL | Enhanced chemi-luminescence |
| dGTP | 2′-Deoxyguanosine 5′-triphosphate |
| DNA | Deoxyribonucleic acid |
| dNTP | 2′-Deoxyribonucleoside 5′-triphosphate |
| DTT | Dithiothreitol |
| dTTP | 2′-Deoxythymidine 5′-triphosphate |
| EDTA | Ethylenediaminetetraacetic acid |
| Gal | Galactose |
| GalNAc | N-acetylgalactosamine |
| Glc | Glucose |
| GLcNAc | N-acetylglucosamine |
| hr | Hour |
| HCl | Hydrogen chloride |

| | |
|---|---|
| $H_2O$ | Water |
| IPTG | Isopropylthio-ß-D-galactoside |
| kb | Kilobase pair |
| kDa | Kilodalton |
| KCl | Potassium chloride |
| *LacZ* | ß-Galactosidase gene |
| M | Molar |
| $\mu$Ci | MicroCurie |
| $\mu$g | Microgram |
| mg | Milligram |
| $MgCl_2$ | Magnesium chloride |
| min | Minute |
| $\mu$l | Microlitre |
| ml | Millilitre |
| $\mu$M | Micromolar |
| mM | Millimolar |
| Mr | Molecular weight |
| mRNA | Messenger ribonucleic acid |
| 4-MU | 4-Methylumbelliferone |
| 4-MU-ß-gal | 4-Methylumbelliferyl ß-D-galactoside |
| 4-MUG | 4-Methylumbelliferyl ß-D-N-acetylglucosamine |
| 4-MUGS | 4-Methylumbelliferyl ß-D-N-acetylglucosamine-6-sulphate |

| | |
|---|---|
| NAN | N-acetylneuraminate |
| NaCl | Sodium chloride |
| NaOH | Sodium hydroxide |
| ng | Nanogram |
| nM | Nanomolar |
| PBS | Phosphate buffered saline |
| PI | Isoelectric pH |
| pmol | Picomole |
| SDS | Sodium dodecyl sulfate |
| SDS-PAGE | SDS-polyacrylamide gel electrophoresis |
| TEMED | N,N,N′,N′-tetramethylethylenediamine |
| Tris-HCl | Tris(hydroxymethyl)aminomethane hydrochloride |
| Volts | Voltages |
| X-gal | 5-Bromo-4-chloro-3-indolyl-ß-D-galactoside |

# 1. INTRODUCTION

Lysosomal ß-hexosaminidase A (Hex A), together with the $G_{M2}$ activator protein, is required for the *in vivo* hydrolysis of $G_{M2}$ ganglioside. Deficiency of this enzyme activity results in Tay-Sachs disease. In Tay-Sachs disease prevention programs designed to identify disease carriers and offer prenatal diagnosis, some individuals who are clinically normal, but deficient in Hex A activity toward synthetic substrates (ie. Hex A pseudodeficient or Hex A-minus normal), have been found (Vidgoff *et al.*, 1973; Kelly *et al.*, 1976; O'Brien *et al.*, 1978; Thomas *et al.*, 1982; Grebner *et al.*, 1986; Navon *et al.*, 1986; Triggs-Raine et al., 1992). Mutations, C739T(R247W) and C745T(R249W), associated with Hex A pseudodeficiency, and therefore called benign mutations, have been identified (Triggs-Raine *et al.*, 1992; Cao *et al.*, 1993). The study of benign mutations was an essential task because their molecular and biochemical nature was unknown and the screening methods could not differentiate carriers of benign mutations from carriers of disease-causing mutations. This could result in misleading counselling and possibly, incorrect prenatal diagnoses. The high frequency (~38%) of benign mutations in enzyme-defined non-Jewish carriers (Cao *et al.*, 1993) further necessitated the study of these mutations.

## 1.0 History of Tay-Sachs disease.

Tay-Sachs disease was named after its discoverers, who described the fundamental clinical and pathological characteristics of this prototype of $G_{M2}$ gangliosidosis. Dr. Warren Tay was a British ophthalmologist who observed a cherry-red spot in the retina of an infant patient with mental and physical retardation (Tay, 1881). Dr. Bernard Sachs was an American neurologist who found the enlarged cytoplasm of neurons as a histopathological

characteristic of the disease. Sachs also noted the familial nature of cases and the prevalence of the disease in Jews (Sachs, 1887 and 1896). The distended neuronal cytoplasm from patients of Tay-Sachs disease revealed, at an ultrastructural level, membranous cytoplasmic bodies, presumably formed from deposited materials. The metabolic basis of the disease was shown to affect a class of acidic ganglionic glycolipids (gangliosides) (Klenk, 1935; 1939 and 1942). These deposited materials were identified primarily as ganglioside $G_{M2}$ (GalNAc-ß(1-4)-[NAN-α(2-3)-]-Gal-ß(1-4)-Glc-ceramide) (Svennerholm, 1962; Makita and Yamakawa, 1963; Ledeen and Salsman, 1965). A primary accumulation of $G_{M2}$ ganglioside caused by a defect in the hydrolytic degradation of this compound can cause the disease, $G_{M2}$ gangliosidosis (Suzuki and Chen, 1967; Neufeld, 1989; Gravel *et al.*, 1995). The deficiency of ß-hexosaminidase A as the biochemical basis of $G_{M2}$ gangliosidosis was indicated by the finding that the accumulated $G_{M2}$ ganglioside had a terminal residue of ß-N-acetylgalactosamine. This was verified by Okada and O'Brien (1969) who showed the absence of ß-hexosaminidase A in a Tay-Sachs disease patient using the protocol described by Robinson and Stirling (1968) to separate ß-hexosaminidase A (heat labile and acidic, PI = 5.4) from B (heat stable and basic, PI = 7.9) by gel electrophoresis.

## 1.1 The biological function of ß-hexosaminidase.

*In vivo*, both hexosaminidase A and B catalyze the cleavage of the terminal non-reducing sugar, GlcNAc or GalNAc, at the glycosidic bond in ß-configuration, away from most glycoproteins, oligosaccharides, glycosaminoglycans and glycolipids (Gravel *et al.*, 1995). Hex A, with the assistance of the $G_{M2}$ activator protein (a *GM2A* gene product), is

the only enzyme that can hydrolyse the terminal GalNAc from $G_{M2}$ ganglioside *in vivo* (Conzelmann and Sandhoff, 1979). Two models (review by Sandhoff *et al.*, 1995) were postulated for the participation of the $G_{M2}$ activator protein in *in vivo* $G_{M2}$ ganglioside hydrolysis: (1) The $G_{M2}$ activator protein solubilizes $G_{M2}$ ganglioside from the lysosomal membrane and presents it, in a 1:1 ratio complex, to the water-soluble enzyme, Hex A, for hydrolysis (Conzelmann and Sandhoff, 1979). This hypothesis was supported by an *in vitro* experiment showing that the activator protein can transfer labelled gangliosides from one liposome to another (Conzelmann *et al.*, 1982). Studies by Li *et al.* (1995) have shown that the $G_{M2}$ activator protein specifically recognizes and interacts with the trisaccharide structure of the $G_{M2}$ epitope and presents the GalNAc residue to Hex A for hydrolysis. (2) The activator protein lifts a $G_{M2}$ ganglioside molecule partially out of the lysosomal membrane so that the ganglioside becomes accessable for Hex A to bind for hydrolysis (Furst and Sandhoff, 1992).

1.2 ß-Hexosaminidase isoenzymes.

ß-Hexosaminidase A, or Hex A (acidic), is one of the major isoenzymes of ß-hexosaminidase (ß-N-acetyl-D-hexosaminidase, EC 3.2.1.52); the other major isoenzyme is ß-hexosaminidase B, or Hex B (basic). The remaining isoenzymes, ß-hexosaminidase S, or Hex S, ß-hexosaminidase I (P), or Hex I (P), and ß-hexosaminidase D, or Hex D (Mahuran *et al.*, 1985) are normally minor forms.

Hex A, an acidic isoenzyme, is comprised of a dimer of one α-subunit encoded by the *HEXA* gene and one ß-subunit encoded by the *HEXB* gene. Hex B, a basic isozyme, is a ß-

/ß-subunit dimer and Hex S is an α-/α-subunit dimer. Hex S exists in a very small amount in physiological conditions, but it is higher in certain cell types (Emiliani *et al.*, 1990) and in Sandhoff disease patients where the ß-subunit is absent. Hex I has an intermediate PI and is therefore called the intermediate form of ß-hexosaminidase. Two types of Hex I, Hex $I_1$ and Hex $I_2$, exist; they can be differentiated by their order of elution from an anion exchange column (Mahuran *et al.*, 1985). Serum Hex I or Hex P, found at an elevated level in pregnant women, was shown on the basis of antigenicity to be comprised of a mixture of mature and precursor ß-subunits (Stirling, 1972; Geiger *et al.*, 1978). The tissue Hex I form, intracellular Hex $I_2$, was found to be comprised of partially processed Hex A (Dewji *et al.*, 1986; Mahuran, 1990). Hex D is comprised of two large polypeptides, 71 kDa and 80 kDa, with native molecular mass of 140 kDa; the two polypeptides share almost identical peptide maps suggesting that they may differ in, more likely, carbohydrate composition (Mahuran *et al.*, 1985). However, the two polypeptides are not related to either the α-subunit or the ß-subunit (Mahuran *et al.*, 1985). The 71 kDa polypeptide has activity toward chondroitin- and keratan sulfate-derived substrates (Mahuran *et al.*, 1985). The remainder of this section focuses on the α-subunit containing isoenzymes because the α-subunit contains the benign mutations associated with Hex A pseudodeficiency and the disease-causing mutations associated with $G_{M2}$ gangliosidosis variant B (including Tay-Sachs disease). Some aspects of the ß-subunit which are relevant to these studies, are also discussed.

## 1.3 The *HEXA* gene.

*HEXA,* encoding the α-subunit of Hex A, has been located to human chromosome

15q23→24 (Takeda *et al.*, 1990; Nakai *et al.*, 1991). The gene is made up of 14 exons which span 35 kb (Proia and Soravia, 1987). The α-subunit cDNA contains an open reading frame of 1587 base pairs that encodes a protein made of 529 amino acid residues (Myerowitz *et al.*, 1985; Korneluk *et al.*, 1986). A region of human *HEXA* with promoter function was located between 60 and 100 bp upstream of the initiating ATG codon; it showed features of a housekeeping promoter. This region did not contain CAAT and TATA boxes that are typically present in regulated genes, but, contained multiple initiation sites. In addition, human *HEXA* gene transcripts, analysed by Northern blot, were shown to be ubiquitously expressed (Norflus *et al.*, 1996). Interestingly, biochemical studies have suggested that *HEXA* expression might be regulated (Swank *et al.*, 1978, Emiliani *et al.*, 1992, Rahi and Srivastava, 1983, and Stirling *et al.*, 1991). Indeed, the promoter region contained two potential half-sites of an estrogen response element and potential binding sites for transcription factors NF-E1 and AP-2 (Norflus *et al.*, 1996).

The promoter region of the mouse *hexa* gene was also found in the 150 bp region upstream of the initiator ATG (Wakamatsu *et al.*, 1994). The critical region for the expression of the mouse gene was narrowed to a region between 145 and 75 bp upstream of the initiation coden ATG (Norflus *et al.*, 1996). The promoter regions of human *HEXA* and mouse *hexa* showed strong similarity in the region between 96 bp upstream and the ATG initiation site, indicating the importance of this region for the expression of the gene (Norflus *et al.*, 1996).

Analysis of sequence alignments showed that the human *HEXA* gene shared 55% nucleotide and 57% amino acid homology with the human *HEXB* gene (Korneluk *et al.*,

1986). Comparison of these two genes demonstrated that the positions of the splice sites were homologous for 12 (of 13) intron/exon junctions (Proia, 1988; Neote *et al.*, 1988). This indicated a common ancestral origin of these two genes.

1.4 Biosynthesis and processing of ß-hexosaminidase A.

Both the α- and ß-subunits of Hex A (αß) are synthesized at the rough endoplasmic reticulum (rER), transported through the Golgi and targeted to the lysosome or secreted extracellularly (reviewed in Neote at al., 1991). The signal peptides at the N-terminus of the proteins function to lead the nascent peptide into the lumen of the ER and are cleaved during the translocation process (reviewed by von Heijne, 1990a; 1990b). The prepro-α-subunit (529 residues) has a signal peptide of 22 amino acids (Little *et al.*, 1988) and the prepro-ß-subunit (556 residues) has a signal peptide of 42 amino acids (Stirling *et al.*, 1988; Quon *et al.*, 1989). The removal of the signal peptide results in a pro-α-subunit of 507 amino acids with a molecular mass of about 67 kDa and a pro-ß-subunit of 514 amino acids with a molecular mass of about 63 kDa.

Glycosylation of the α-subunit peptide occurs co-translationally upon entry into the lumen of the ER. This process includes the transfer of a mannose-rich moiety, $Glc_3Man_9GlcNAc_2$, presented by the carrier lipid dolichol molecule, to the $NH_2$ group on the side chain of specific Asn residues within the sequence Asn-X-Ser/Thr, where X can be any amino acid except Pro or Asp (Kornfeld and Kornfeld, 1985). This reaction is catalyzed by a membrane-bound oligosaccharyl transferase with the active site facing the lumen of the ER. The pro-α-chain has three glycosylation sites, Asn115, 157 and 295, all of which are

glycosylated in cultured fibroblasts (Weitz and Proia, 1992). The pro-ß-chain has five glycosylation sites located at Asn84, 142, 190, 327, and 497; the first four sites were shown to be glycosylated in the ß-subunit as Hex A and B in placenta and as Hex B in transfected Cos cells (O'Dowd *et al.*, 1988; Sonderfeld-Fresko and Proia, 1989).

Processing of the oligosaccharide chains begins in the ER with the removal of the terminal three glucosides by glucosidases. The exposed mannose residues are subjected to phosphorylation at the 6th position of the mannose residue by the sequential action of two enzymes. UDP-GlcNAc : N-acetylglucosamine-1-phosphotransferase (phosphotransferase) transfers GlcNAc-1-phosphate of UDP-GlcNAc, in the late ER and cis Golgi apparatus (Pelham, 1989; Semenza *et al.*, 1990), to a mannose unit on the oligosaccharide (Lang *et al.*, 1984). N-acetylglucosamine-1-phosphodiester-α-N-acetylglucosaminidase then removes the terminal GlcNAc to expose a mannose-6-phosphate residue in the late Golgi apparatus (Varki and Kornfeld, 1980). Phosphorylation is required for the normal function of Hex A (Proia *et al.*, 1984; reviewed by Kornfeld, 1990). In the absence of phosphorylation, ß-hexosaminidase fails to reach the lysosomes, resulting in a deficiency of this enzyme activity, as observed due to a defect in the phosphotransferase in patients with I-cell disease (Hasilik *et al.*, 1981). However, a normal level of ß-hexosaminidase was observed in the isolated lysosomes from I-cell lymphoblasts (Miller *et al.*, 1993) suggesting that this cell type may target the lysosomal enzymes to the lysosome via a mannose-6-phosphate independent pathway (Glickman and Kornfeld, 1993).

Proper folding of the α- and ß-subunits in the ER is also important for normal processing (reviewed by Hurtley and Helenius, 1989; Pelham, 1989; Matthews, 1993). It was

shown that the mutation in the α-subunit, G250D, associated with juvenile-onset $G_{M2}$ gangliosidosis (Hechtman *et al.*, 1989; Trop *et al.*, 1992), appeared to alter the folding of the α-subunit, producing an insoluble protein. The phosphorylation process requires a properly folded α- or ß-subunit. It was found that the phosphotransferase phosphorylated lysosomal enzymes by recognizing a specific properly-folded protein domain with the mannose moiety of a oligosaccharide nearby (Lang *et al.*, 1984; Cantor and Kornfeld, 1992). A properly folded protein is also required for dimerization of the α- and ß-subunits.

Dimerization of the two subunits is required for normal ß-hexosaminidase function. It was shown that only the subunit dimers, and not the monomers, possessed ß-hexosaminidase activity (Proia *et al.*, 1984). It was also suggested that dimerization of the α-subunit with the ß-subunit was a prerequisite for transport of the α-chain to the lysosome (Proia *et al.*, 1984). The subcellular localization of αß subunit dimerization is controversial. It was predicted that the dimerization of the α- and ß-subunit occured in the Golgi apparatus because the α- and ß-subunit precursors were apparently phosphorylated before the formation of an αß dimer (Proia *et al.*, 1984) and phosphotransferase activity responsible for the phosphorylation was shown to be present in late ER and cis Golgi apparatus (Lazzarino and Gabel, 1988; Pelham, 1989; Semenza *et al.*, 1990). However, it seems unlikely that phosphorylation precedes dimerization since the phosphotransferase recognizes a tertiary structure (Lang *et al.*, 1984; Cantor and Kornfeld, 1992). Further, it was shown that in fibroblasts monomeric α-subunit could also be secreted (Proia *et al.*, 1984).

After the α- and ß-subunits are dimerized and phosphorylated, the enzyme is transported to the lysosome. A phosphorylated mannose residue(s), that is the mannose-6-

phosphate moiety on Hex A, functions as a recognition marker. It lends Hex A high affinity for the mannose-6-phosphate receptor (Dahm *et al.*, 1989), that is present in the *tans*-Golgi apparatus where the association of the receptor and Hex A occurs (Kornfeld, 1992). The Hex A-receptor complex is then transferred via coated vesicles either directly to the lysosome or to prelysosomal compartments which will mature to become lysosomes (Croze *et al.*, 1989; Kornfeld, 1990; Rothman and Wieland, 1996). In the acidic environment of the lysosome, the receptors dissociate the ligand, Hex A, and recycle back to the Golgi apparatus to deliver additional ligands. In normal conditions, some Hex A molecules which are not phosphorylated, or phosphorylated but not bound to the mannose-6-phosphate receptor, are secreted into body fluid or the medium of cultured cells. With $NH_4Cl$ treatment, secretion of Hex A can be enhanced in cultured cells (Seglen, 1983).

Inside the lysosome, ß-hexosaminidase undergoes a maturation process that includes glycosidic and proteolytic hydrolysis. The glycosidic process removes the mannose-6-phosphate moiety and partially degrades the carbohydrate residues (O'Dowd *et al.*, 1988). The proteolytic cleavage of a pro-Hex A (pro-α/pro-ß dimer) results in a mature Hex A which is comprised of $\alpha_p\alpha_m$ /$ß_pß_bß_a$ where the five polypeptides are linked by disulphide bonds (Mahuran, 1990; O'Dowd *et al.*, 1988). The biological significance of the maturation process is not clear since the modifications in the lysosome do not appear to alter the catalytic activity of Hex A compared with the precursor form in the serum (Zuhlsdorf *et al.*, 1983). The capacity of precursor Hex A for $G_{M2}$ ganglioside hydrolysis *in vivo* is unknown. For an analysis of Hex A, the precursor and mature α- and ß-subunits can be differentiated by SDS-polyacrylamide gel electrophoresis. The forms of the α-subunit include a 67 kDa precursor,

a 54 kDa mature ($\alpha_m$) (Hasilik and Neufeld, 1980a; Mahuran and Lowden, 1980), and a 7 kDa peptide($\alpha_p$) (Hubbes *et al.*, 1989). The ß-subunit forms include a 63 kDa precursor, a 52 kDa intermediate form, 28 kDa and 24-26 kDa $ß_b$ peptides of the mature ß-subunit (Mahuran *et al.*, 1982 and 1988; Quon *et al.*, 1989), and the 7-10 kDa $ß_p$ peptide (Hubbes *et al.*, 1989; Quon *et al.*, 1989; Sonderfeld-Fresko and Proia, 1989).

1.5 $G_{M2}$ gangliosidosis variants.

Normal hydrolysis of $G_{M2}$ ganglioside requires three gene products; they are the α-subunit, ß-subunit and $G_{M2}$ activator protein encoded by *HEXA*, *HEXB* and *GM2A* genes, respectively. Mutations in any of these genes, when present in the homozygous state, may result in $G_{M2}$ gangliosidosis. $G_{M2}$ gangliosidosis has been classified into three variants based on which isoenzyme remains in the tissues of the affected patient (Sandhoff *et al.*, 1971). If Hex A deficiency results from a defect in the α-subunit, it is called $G_{M2}$ gangliosidosis variant B because patients still have normal or elevated levels of Hex B activity; Tay-Sachs disease is an example. If there is a ß-subunit defect, it is called $G_{M2}$ gangliosidosis variant O because neither Hex A (αß) nor Hex B (ßß) activity are detectable. An example of such a disease is Sandhoff disease. A deficiency in Hex A activity that results from a defect in the $G_{M2}$ activator protein is called $G_{M2}$ gangliosidosis variant AB because both Hex A and Hex B are normally expressed and their activities are in the normal range when measured using synthetic substrates. However, the *in vivo* $G_{M2}$ ganglioside hydrolysis is impaired. Although other nomenclatures for $G_{M2}$ gangliosidosis have also been proposed and used (Sandhoff *et al.*, 1989; Gravel *et al.*, 1995), the nomenclature proposed by Sandhoff *et al.* (1971) is applied

in this thesis.

1.6 Clinical heterogeneity of $G_{M2}$ gangliosidosis variant B.

$G_{M2}$ gangliosidosis has been arbitrarily classified, based on the onset stage of disease, into infantile, juvenile and adult forms. Tay-Sachs disease is the infantile form of $G_{M2}$ gangliosidosis. The clinical manifestations vary widely from infantile Tay-Sachs disease to adult-onset $G_{M2}$ gangliosidosis (see review by Gravel *et al.*, 1995). It has been observed that the development of symptoms is delayed in less severe forms of the disease. The severity of the clinical phenotype of $G_{M2}$ gangliosidosis was documented to be inversely correlated with the residual Hex A activity (Conzelmann *et al.*, 1983; Leinekugel *et al.*, 1992). Mutations in the α-subunit associated with the various forms of $G_{M2}$ gangliosidosis have been identified (see the following section). Interestingly, siblings or patients with the same molecular defect in the *HEXA* gene have sometimes manifested different clinical symptoms, indicating that the molecular defects alone cannot fully determine the clinical phenotype (Navon *et al.*, 1986; Kolodny *et al.*, 1983).

Clinically, infants affected with classical Tay-Sachs disease appear normal at birth (Gravel *et al.*, 1995). By 3-5 months of age, motor weakness and heightened startle response may have been evident, but these are not typically noted before the diagnosis. Progressive weakness and hypotonia become obvious by 6-10 months of age. In some cases, blindness and unusual eye movements also raise parental concern. The "cherry red spot" is a classical sign of all Tay-Sachs disease. As the disease progresses, seizures become common, the patients become less responsive to the surroundings, and death usually occurs before 4-years

of age.

For the juvenile or subacute form of $G_{M2}$ gangliosidosis, clinical abnormalities typically appear between 2 and 10 years of age. The predominant features are dementia and developmental regression, particularly in speech and acquired life skills. Loss of vision, development of seizures, and increasing spasticity are also characteristics (Brett *et al.*, 1973).

For adult-onset and chronic $G_{M2}$ gangliosidosis, the clinical phenotype is more variable because symptoms associated with different parts of the central nervous system dominate in different individuals. The symptoms often include progressive dystonia, spinocerebellar degeneration, motor neuron disease and psychosis (Johnson, 1981; Federico *et al.*, 1991).

1.7 Molecular basis of $G_{M2}$ gangliosidosis variant B.

Approximately 54 mutations in the *HEXA* gene have been identified, to date, in DNA samples from patients with various forms of $G_{M2}$ gangliosidosis. These include deletion, insertion and base substitution mutations (Gravel *et al.*, 1995). The mechanism by which the mutations lead to Hex A deficiency can be summarized as: (1) No mRNA or highly unstable mRNA is made, and as a result neither the α-subunit nor Hex A activity are produced, e.g a 4-bp insertion in exon 11 (Myerowitz and Costigan, 1988). These mutations are always associated with early-onset forms of $G_{M2}$ gangliosidosis; (2) Stable mRNA and a certain amount of mutant enzymatic protein, and therefore residual Hex A activity, are produced, e.g. a base substitution G805A (G269S) (Paw *et al.*, 1989; Navon and Proia, 1989). These mutations were primarily found in late-onset and slow progressing forms of the disease.

Of the 54 described mutations, most are associated with Tay-Sachs disease and are

not common mutations. A few of these mutations have a high frequency in specific ethnic groups and those mutations have been the subject of the most intensive studies (Gravel *et al.*, 1995). The 4-bp insertion in exon 11, $+TATC_{1278\text{-}1281}$ (Myerowitz and Costigan, 1988), results in Tay-Sachs disease and accounts for approximately 81% of mutant alleles in the Ashkenazi Jewish population (Paw *et al.*, 1990; Triggs-Raine *et al.*, 1990; Grebner and Tomczak, 1991; Landels *et al.*, 1991; Fernandes *et al.*, 1992a). The *HEXA* gene with this mutation was shown to be normally transcribed, but the mRNA was not detectable by Northern blot indicating it was unstable (Paw and Neufeld, 1988; Nishimoto *et al.*, 1991; Boles and Proia, 1995). A second mutation frequent among Ashkenazi Jews is the donor splice-junction mutation, intron 12 +1 G→C (Arpaia *et al.*, 1988; Myerowitz, 1988; Ohno and Suzuki, 1988a and 1988b). This mutation is also associated with the infantile form of the disease and accounts for about 15% of mutant alleles in this population (Paw *et al.*, 1990; Triggs-Raine *et al.*, 1990; Grebner and Tomczak, 1991; Landels *et al.*, 1991; Fernandes *et al.*, 1992a; Kaback *et al.*, 1993). This mutation disrupts normal mRNA splicing, leading to a diverse array of abnormally spliced mRNA products (Ohno and Suzuki, 1988a). A third mutation common in this ethnic group is a single base substitution at the 3' end of exon 7, G805A (G269S) (Navon and Proia, 1989; Paw *et al.*, 1989), which is associated with the adult-onset form of the disease; it accounts for about 2% of the disease alleles in this population (Paw *et al.*, 1990; Triggs-Raine *et al.*, 1990; Grebner and Tomczak, 1991; Fernandes *et al.*, 1992a; Kaback *et al.*, 1993). These mutations are not completely restricted to the Ashkenazi Jewish population as both the 4-bp insertion and G805A mutations have been found in non-Jewish populations (Navon *et al.*, 1990).

In French Canadian Tay-Sachs disease patients, a 7.6-kb deletion at the 5' end of the *HEXA* gene is the predominant mutant allele. This deletion starts about 2 kb upstream of exon 1 and extends into intron 1, resulting in the absence of α-subunit mRNA and its protein. It accounts for about 80% of the disease alleles in this population (Myerowitz and Hogikyan, 1986 and 1987; Hechtman *et al.*, 1990; Triggs-Raine *et al.*, 1995).

A base substitution, C508T in exon 5, resulting in an amino acid change from Arg to Trp at position 170, was also found in a French Canadian Tay-Sachs disease patient who was a compound heterozygote for this mutation and a B1 (D258H) mutation (see following section for B1 mutations) (Fernandes *et al.*, 1992b). This C508T (R170W) mutation was also found by Akli *et al.* (1993b) in an Italian patient and by Triggs-Raine *et al.* (1995) in a Franco-American carrier. Expression studies for this mutation have not been done.

In the non-Jewish population, IVS-9 + 1 G→A was identified to be a common Tay-Sachs disease mutation (Akli *et al.*, 1991 and 1993a) with a frequency of 14% (9/64) among enzyme-defined carriers (Akerman *et al.*, 1992; Landels *et al.*, 1992 and 1993; Kaback *et al.*, 1993). This mutation results in aberrantly spliced mRNA and no expressed protein.

In addition to the more frequently occurring mutations, one "private" mutation was used in this study as a control. This was the G749A (G250D) mutation in exon 7 that produced an unstable unphosphorylated α-subunit (Hechtman *et al.*, 1989). It is associated with the juvenile form of $G_{M2}$ gangliosidosis (Andermann *et al.*, 1977; Hechtman *et al.*, 1989; Trop *et al.*, 1992). Expression of Hex A harbouring this mutation in Cos-7 cells showed that the levels of expressed activity were about 12% of the wild type and expressed Hex S was not detectable (Trop *et al.*, 1992).

1.8 Adult-onset $G_{M2}$ gangliosidosis and the G269S mutation.

The clinical manifestations of adult-onset $G_{M2}$ gangliosidosis typically become evident between twenty and thirty years of age, and are less severe than the symptoms associated with other forms of $G_{M2}$ gangliosidosis (Navon *et al.*, 1986). A G805A(G269S) mutation in the α-subunit of Hex A was identified in several patients with this disorder (Navon and Proia, 1989; Paw *et al.*, 1989). Most adult-onset $G_{M2}$ gangliosidosis patients were found to be compound heterozygotes for the G269S mutation and one of the common Tay-Sachs disease mutations (Navon and Proia, 1989; Paw *et al.*, 1989). The G805A mutation falls at the last position of exon 7 and is associated with a reduced level (~15% of normal) of α-chain mRNA (Paw *et al.*, 1989; Navon and Proia, 1989), a decreased level of α-subunit precursor (Frisch *et al.*, 1984; d'Azzo *et al.*, 1984), a low level of activity toward $G_{M2}$ ganglioside (Conzelmann *et al.*, 1983), and a level of activity toward the synthetic substrate in the Tay-Sachs disease range (Navon *et al.*, 1980 and 1986). Some concern that the G269S mutation might also be associated with the Hex A pseudodeficiency phenotype has also been expressed (Kappler *et al.*, 1990). This possibility was ruled out, and the G269S mutation was confirmed as a primary cause of adult-onset $G_{M2}$ gangliosidosis by Navon *et al.* (1990). They also found adult $G_{M2}$ gangliosidosis patients who were homozygous for this mutation. This clearly demonstrated the relationship between the G269S mutation and the adult-onset form of the disease (Proia *et al.*, 1990).

The effect of the G269S mutation on the α-subunit of Hex A was studied in fibroblasts and as expressed Hex S and Hex A in Cos-7 cells. It was shown using fibroblasts that the α-subunit harbouring this mutation failed to associate with the ß-subunit and could not be

converted from the precursor form to the mature form (d'Azzo *et al.*, 1984). Further analysis using Cos-7 cells in an *ex vivo* expression system suggested that the G269S mutation affects the dimerization of the α- and ß-subunit indirectly by destabilizing the α-subunit (Brown and Mahuran, 1993). Using the Cos-7 expression system, it was shown that the G269S mutation produced almost no Hex S activity, but 46% of the normal Hex A activity when the α- and ß-subunits were co-transfected (Navon and Proia, 1989; Brown and Mahuran, 1993). This indicated that the mutant α-subunit was stabilized by the ß-subunit. The late-onset clinical course associated with this mutation, and the substantial level of expressed Hex A activity in transfected Cos-7 cells, showed that the G269S mutation could produce a substantial level of Hex A activity, albeit lower than the critical threshold required for normal levels of $G_{M2}$ ganglioside hydrolysis (Conzelmann *et al.*, 1983).

1.9 B1 mutations and implications for the α-subunit active site.

$G_{M2}$ gangliosidosis variant B1 refers to Tay-Sachs disease patients who have a normal level of α-subunit protein and Hex A activity toward the synthetic substrate 4-MUG, but a deficiency of Hex A activity toward 4-MUGS or $G_{M2}$ ganglioside (Ohno and Suzuki, 1988c; Suzuki and Vanier, 1991). The mutations associated with the variant B1 phenotype are called B1 mutations. To date, the molecular basis of variant B1 forms have included four B1 mutations, R178H (Ohno and Suzuki, 1988c; Santos *et al.*, 1991; Tanaka *et al.*, 1990), R178C (Tanaka *et al.*, 1990), R178L (Triggs-Raine *et al.*, 1991) and D258H (Fernandes *et al.*, 1992b) in the α-subunit. The biochemical phenotype associated with B1 mutations can be explained by the fact that they do not interfere in the synthesis, processing, dimerization

or targeting of Hex A to the lysosome. Instead, they disrupt the catalytic site on the α-subunit, and the normal Hex A activity to 4-MUG is derived from the normal ß-subunit of Hex A (Kytzia *et al.*, 1983). This suggests that the B1 residues should be part of the catalytic site in the α-subunit. Mutations in codon 178 disrupt the catalytic activity of the α-subunit (Brown and Mahuran, 1991). This residue was shown to be conserved in the human and mouse α- and ß-subunits and the ß-hexosaminidase of *Dictyostelium discoideum* (Brown *et al.*, 1989). A structural model of human Hex A, based on the crystal structure of chitobiase, suggested that the residues R178 and D258 are located in the central pocket of the active site (Tews *et al.*, 1996) as might be expected from their B1 phenotype.

1.10 Measurements of Hex A activity.

Hex A activity can be measured by using both the natural substrate, $G_{M2}$ ganglioside, and synthetic substrates. An *in vitro* assay using labelled $G_{M2}$ ganglioside as a substrate is a reliable method (Conzelmann *et al.*, 1983). However, this assay is not commonly used in laboratories because labelled $G_{M2}$ ganglioside is currently not commercially available and it is difficult to prepare, the turnover rate of the substrate to the product, $G_{M3}$ ganglioside, is low and the reaction requires the presence of $G_{M2}$ activator protein which is also difficult to prepare. Alternatively, detergents such as sodium taurocholate can replace the $G_{M2}$ activator protein for the $G_{M2}$ hydrolysis by Hex A in an *in vitro* assay (O'Brien *et al.*, 1977; Harzer, 1983), but the determined activity is not specific to Hex A because Hex B and Hex S can also hydrolyse $G_{M2}$ ganglioside in the presence of detergents (Erzberger *et al.*, 1980). An alternative approach to determine Hex A activity using the natural substrate is an *in situ* assay

according to the procedure by Raghavan *et al.* (1985a; 1985b) and Sonderfeld *et al.* (1985). In this method sphingosine-labelled $G_{M2}$ ganglioside, or $G_{M1}$ ganglioside (Schmid *et al.*, 1992), was fed to the cultured skin fibroblasts. After incubation for about 10 days, lipid extracts were prepared from the cells with chloroform-methanol (2:1). The unhydrolyzed radioactive $G_{M2}$ ganglioside was recovered from the (upper) aqueous phase by adding water and the radioactive product, metabolized $G_{M3}$ ganglioside, was retained in the lower organic phase. An advantage of using this assay system is that the $G_{M2}$ activator protein is not required because it is provided by the endogenous fibroblast $G_{M2}$ activator protein. The disadvantage of this method is that the hydrolytic activity will always appear in a similar range if it is above the threshold required for normal $G_{M2}$ ganglioside hydrolysis.

Two synthetic fluorogenic substrates, 4-methylumbelliferyl ß-D-N-acetylglucosamine (4-MUG) and its sulphated derivative 4-methylumbelliferyl ß-N-acetylglucosamine-6-sulphate (4-MUGS), are commonly used for the measurement of Hex A activity. Because these substrates produce a fluorescent product, the assays are sensitive, do not require $G_{M2}$ activator protein for activity and the procedure is simple (Kaback *et al.*, 1977 b; Bayleran *et al.*, 1984). The synthetic substrate, 4-MUG, can be hydrolysed by Hex A, B and S; however, 4-MUGS, because of the introduction of a negative charge by the sulphate in this compound, can only be hydrolysed by α-subunit-containing Hex A and S (Kresse *et al.*, 1981; Inui and Wenger, 1984). Using 4-MUG as a substrate, the activities of Hex A and S, which are heat sensitive, can be distinguished from heat stable Hex B by a heat treatment (Kaback *et al.*, 1977b).

Recently, Agmon *et al.* (1996) introduced a fluorescent sulforhodamine derivative of

$G_{M2}$ ganglioside (SR12-GM1) as a substrate for an *in situ* assay of Hex A. The levels of the hydrolytic products, intracellular SR12-ceramide and secreted SR12-sphingomyelin, were examined. The substrate, SR12-GM1, has some advantages over $G_{M2}$ ganglioside: (1) SR12-GM1 can be administrated into not only fibroblasts, but also white blood cells (lymphocytes, monocytes and macrophages), $G_{M2}$ ganglioside has currently been tested only in fibroblasts; (2) SR12-GM1 is a fluorogenic rather than radioactive compound. Using SR12-GM1 as a substrate, Hex A activity in fibroblasts from subjects with different forms of $G_{M2}$ gangliosidosis, ie. infantile, adult-onset, and Hex A pseudodeficient subjects, were clearly differentiated. Hex A activity in monocytes from a carrier of adult $G_{M2}$ gangliosidosis and a carrier of Tay-Sachs disease also showed a difference (Agmon *et al.*, 1996), although further studies and a larger number of samples may be required to confirm the finding.

1.11 Substrate specificity and properties of ß-hexosaminidase.

Both Hex A and Hex B are able to hydrolyse naturally existing substances including glycoproteins, oligosaccharides, glycosaminoglycans and glycolipids. However, only Hex A can hydrolyse $G_{M2}$ ganglioside. The basis of the difference in the substrate specificity of Hex A and B is thought to be in the differing activities of the α- and ß-subunits (Kytzia and Sandhoff, 1985). The ß-subunit possesses a catalytic site that hydrolyses neutral, water-soluble substrates, eg. oligosaccharides and the synthetic substrate 4-MUG. Therefore Hex B (ßß) is able to hydrolyse these substrates. The α-subunit possesses the active site toward the substrates with negative charge, such as $G_{M2}$ ganglioside and a synthetic substrate 4-MUGS. Because Hex A is comprised of α- and ß-subunits, it can hydrolyse both neutral and

negatively charged substrates (Kytzia and Sandhoff, 1985). The fact that $G_{M2}$ ganglioside can only be hydrolysed by Hex A (αß), and not Hex S (αα) or Hex B (ßß), suggested that the ß-subunit, together with the α-subunit, are required for the binding and hydrolysis of the $G_{M2}$ ganglioside/$G_{M2}$ activator protein complex. The hydrolysis of the synthetic substrate 4-MUGS by either Hex A or Hex S was shown to be inhibited by the $G_{M2}$ activator protein (Kytzia and Sandhoff, 1985) indicating that the α-subunit also provides a binding site for the activator protein.

Hex A from various human tissues has been characterized. The $K_m$ values for Hex A from human liver, placenta, spleen and serum, were determined to be in the range of 0.5 to 0.9 mM toward 4-MUG (Kytzia and Sandhoff, 1985; Mahuran *et al.*, 1985; Robinson and Stirling, 1968; Wiktorowicz *et al.*, 1977; Kaback, 1972), and the $K_m$ value of Hex A from human placenta toward 4-MUGS was determined to be 0.31 mM (Kytzia and Sandhoff, 1985). Hex A's optimal pH for hydrolysis was 4.4 (Wiktorowicz *et al.*, 1977; Kaback, 1972) and the isoelectric points were determined to be 5.4 for Hex A and 7.9 for Hex B from human placenta (Srivastava *et al.*, 1974).

## 1.12 Animal model of Tay-Sachs disease.

An animal model of Tay-Sachs disease is required to evaluate therapeutic applications since Tay-Sachs disease is currently an incurable disease. Gene therapy (Suhr and Gage, 1993) and enzyme replacement (Dobrenis *et al.*, 1992) are the potential approaches to a treatment for the disease. To date, no satisfactory Tay-Sachs disease models are available, although transgenic mice have been produced. The first "Tay-Sachs disease" mouse

was made by disrupting the *hexa* gene using homologous recombination (Yamanaka *et al.*, 1994). The resulting mice showed biochemical and neuropathological characteristics of Tay-Sachs disease, ie. deficient in Hex A activity and exhibiting the membranous cytoplasmic bodies observed in some regions of the central nervous system of Tay-Sachs disease patients. However, the mice did not develop abnormal neurological manifestations (Yamanaka *et al.*, 1994). It was postulated that mice have two independent metabolic pathways for $G_{M2}$ ganglioside catabolism, while in humans there is only one, ie. the $G_{M2}$ ganglioside was exclusively hydrolysed by Hex A to become $G_{M3}$. The second pathway in mouse was verified in a mouse model of Sandhoff disease (Sango *et al.*, 1995), ie. ganglioside $G_{M2}$ can be degraded to $G_{A2}$ by sialidase, which is then further hydrolysed by Hex A and Hex B. Therefore, the Tay-Sachs disease mice did not develop any neuronal abnormalities, although accumulation of $G_{M2}$ ganglioside in certain regions of the mouse brain indicated that the traditional metabolic pathway for $G_{M2}$ ganglioside, rather than the second pathway through $G_{A2}$, is also predominant in mice.

## 1.13 Tay-Sachs disease prevention programs.

Tay-Sachs disease prevention programs were initiated in 1970 in Jewish communities of Baltimore, MD, and Washington, DC. They included community-based Tay-Sachs disease education, carrier screening, and genetic counseling programs, aimed at prospectively preventing this disease (Kaback and Zeiger, 1972). These programs were based on the finding that Hex A deficiency resulted in this disease (Okada and O'Brien, 1969), and that the synthetic substrate 4-MUG, could be used to quantitatively measure Hex A and Hex B

activities (Robinson and Stirling, 1968). Hex A was differentiated from Hex B using their difference in thermolability; Hex A is heat-labile and Hex B is heat-stable at 52°C. Hex A activity was determined under precise conditions of temperature, pH, ionic strength, and protein concentration, according to standard procedures (Kaback *et al.*, 1977a; Lowden *et al.*, 1973).

In carrier screening programs, the aims were to identify the disease allele-carriers and therefore carrier couples whose offspring might be at risk for Tay-Sachs disease. Serum and leucocytes were commonly used for carrier screening. However, leucocytes were essential to test in pregnant women because Hex P, or serum Hex $I_2$ , a placental isoenzyme of ß-hexosaminidase, appeared in the maternal serum (Stirling, 1972; Lowden, 1979). Hex P has thermolability similar to Hex B, and therefore the percent Hex A would appear lower using the heat-inactivation method and 4-MUG as the substrate for differentiating Hex A and Hex B activities (Navon *et al.*, 1973). For prenatal diagnosis, amniocentesis or chorionic villus sampling were used to obtain the sample for enzymatic analysis.

The heat-inactivation method using 4-MUG as the synthetic substrate also has some pitfalls. When using this method to determine Hex A activity, Hex A activity appears higher in the sample from a variant form Sandhoff disease patient who has thermolabile Hex B (Momoi *et al.*, 1978), and Hex A activity appears in the heterozygous range in the B1 variant Tay-Sachs disease patients (see section 1.9). In addition, carriers of late-onset forms of $G_{M2}$ gangliosidosis or Hex A pseudodeficiency, cannot be differentiated from carriers of Tay-Sachs disease. These pitfalls and others could be overcome by further analysis using the synthetic substrate 4-MUGS and/or DNA testing.

As a result of these programs, the number of newly diagnosed Tay-Sachs disease patients in the United States and Canada has dropped from about 60 cases/year before 1970 to less than 5 cases/year in 1983 (Kaback *et al.*, 1993).

Based on death-record-derived data, the annual birth incidence of Tay-Sachs disease patients was predicted to be about 1/4,000 among Ashkenazi Jews and 100 times less frequent among non-Jews. The carrier frequency of Tay-Sachs disease was predicted to be about 1/300 in non-Jews and 1/30-1/40 among Ashkenazi Jews (Myrianthopoulos *et al.*, 1967). However, the carrier frequency of Tay-Sachs disease was estimated by an enzyme-screening method to be 1/167 in the non-Jewish population (Kaback *et al.*, 1978) and 1/31 among Ashkenazi Jews (Petersen *et al.*, 1983).

1.14 Lysosomal hydrolase pseudodeficiency.

Deficiency of a lysosomal hydrolase activity is mostly associated with a disease; however, individuals who were clinically normal, but deficient in a lysosomal enzyme activity, have also been found. This phenomenon has been referred to as "pseudodeficiency". Currently, there are nine lysosomal enzymes that are associated with clinical pseudodeficiency phenotypes (review by Thomas, 1994). They are galactosylceramidase (Wenger and Louie, 1991; Desnick *et al.*, 1992), α-galactosidase (Bach *et al.*, 1982; Bishop *et al.*, 1981), ß-glucuronidase (Chabas *et al.*, 1991), α-L-iduronidase (Gatti *et al.*, 1985; Whitley *et al.*, 1987; Taylor and Thomas, 1993), α-glucosidase (Nishimoto *et al.*, 1988), α-L-fucosidase (Ramage and Cunningham, 1975; Wood, 1976; Alhadeff and Andrews-Smith, 1978), arylsulfatase A (Gieselmann *et al.*, 1989 and 1991), ß-hexosaminidase A & B (Dreyfus *et al.*, 1975 and 1977)

and ß-hexosaminidase A (Triggs-Raine *et al.*, 1992; Cao *et al.*, 1993). The biochemical features of most of the enzymes associated with pseudodeficiency phenotypes have not been well studied and their molecular basis is still unknown. Among these enzymes, arylsulfatase A and ß-hexosaminidase A & B pseudodeficiencies have been relatively well studied and these studies are briefly described in this section. A thorough description of Hex A pseudodeficiency follows this section.

Arylsulfatase A hydrolyses 3-sulfogalactosylceramide to release the sulfate moiety. Deficiency of this enzyme activity results in metachromatic leukodystrophy (MLD) (Dulaney and Moser, 1980). Arylsulfatase A pseudodeficient individuals showed low activity toward synthetic substrate. The mutation associated with the pseudodeficiency was indentified to be an A2725G base substitution which results in the loss of the polyadenylation signal and abnormal mRNA species (Gieselmann *et al.*, 1989 and 1991). The second mutation found in the pseudodeficient alleles (pd) was a N350S amino acid substitution which results in the loss of a glycosylation site; however, the second mutation does not affect arylsulfatase activity (Gieselmann *et al.*, 1989; Nelson *et al.*, 1991). Arylsulfatase activity in homozygotes of pd/pd alleles (~10% of normal) is higher than that (<10 % of normal) in the compound heterogygotes of a pd allele and a MLD allele (Chabas et al., 1993). The loading studies using radiolabelled sulfatide and fibroblasts showed that 10-15% of normal arysulfatase A activity is sufficient to hydrolyse sulfatide at normal levels (Leinekugel *et al.*, 1992).

ß-Hexosaminidase A & B pseudodeficiency was observed in a healthy father of two Sandhoff disease patients. He was found to be deficient in both Hex A and Hex B activities measured using the synthetic substrate, but, when the natural substrate was used the activities

were in the Sandhoff disease heterozygote range (Dreyfus *et al.*, 1975 and 1977). The molecular basis of the pseudodeficiency was a duplication of a region spanning the junction of intron 13 and exon 14 of the *HEXB* gene, resulting in an alternative splice site and an 18-bp insertion into the ß-subunit mRNA (Dlott *et al.*, 1990). A small amount of normal mRNA was also observed and responsible for the residual (~10%) ß-hexosaminidase activity. It was shown that approximately 10% of normal Hex A activity was the critical threshold level required for maintaining normal $G_{M2}$ ganglioside hydrolysis *in vivo* (Leinekugel *et al.*, 1992; Conzelmann *et al.*, 1983).

1.15 Hex A pseudodeficiency and benign mutations.

Hex A pseudodeficiency or Hex A-minus normal is used to define those individuals who are clinically normal, but deficient in Hex A activity measured using synthetic substrates. At the molecular level the mutations associated with Hex A pseudodeficiency have been termed benign mutations.

Hex A pseudodeficient subjects were found through screening for carriers of Tay-Sachs disease and during prenatal diagnosis. O'Brien *et al.* (1978) described a 28-year-old healthy male who was found to have very low levels of serum Hex A activity in a Tay-Sachs disease community screening program. Further analysis showed 8% Hex A activity in fibroblasts when measured using the synthetic substrate 4-MUG and 41% of normal Hex A activity when determined using [$H^3$] $G_{M2}$ ganglioside as the substrate in the presence of sodium taurocholate. The patient's mother was of Ashkenazi Jewish origin and his father was of Syrian Sephardic Jewish origin. Vidgoff *et al.* (1973) reported a healthy 29 year-old non-

Jewish mother of a Tay-Sachs disease patient with very low heat labile Hex A activity toward 4-MUG in serum and a leucocyte level similar to the disease level. Hex A activities in the samples from her parents and one offspring were found to be in Tay-Sachs disease carrier range. Kelly *et al.* (1976) presented a healthy 37-year-old non-Jewish female who had low Hex A activity in serum, leucocytes and fibroblasts. She was found when she requested testing at the time of her spouse's testing. Her Hex A activity determined using fibroblast extract and $G_{M2}$ ganglioside as the substrate in the presence of detergent showed a level of hydrolysis similar to that of Tay-Sach disease heterozygote. Further analysis using the fibroblasts from this subject demonstrated normal synthesis, processing and secretion of Hex A (Thomas *et al.*, 1982). Thomas *et al.* (1982) also reported a healthy one-year-old Askenazi Jewish infant who had Hex A with biochemical features similar to that reported by Kelly *et al.* (1976). Grebner *et al.* (1986) reported three unrelated healthy individuals. Two were of Ashkenazi Jewish origin and diagnosed prenatally with very low Hex A activity in amnionic fluid and 8% and 25% Hex A in cultured cells from amnionic fluid. By the age of 2 and 5-years, no Hex A activity was detectable in serum, but low levels of activity toward 4-MUG were evident in leucocytes and fibroblasts. The third individual was a 30-year-old of non-Jewish origin with uniformly low Hex A activity in serum, leucocytes and fibroblasts. Analysis using the fibroblasts from these individuals revealed a normal level of $G_{M2}$ ganglioside hydrolysis using the *in situ* assay, normal synthesis, processing and secretion of Hex A. A 47-year-old Hex A pseudodeficient Ashkenazi Jew, and a 42-year-old non-Jewish Hex A pseudodeficient woman were also described by Triggs-Raine *et al.* (1992) and Cao *et al.* (1993). The biochemical characteristics of the Hex A in samples from these

pseudodeficient subjects are summarized below.

Hex A pseudodeficiency is associated with unique biochemical properties. The two benign mutations in the *HEXA*, C739T(R247W) and C745T(R249W), associated with Hex A pseudodeficiency, have different biochemical phenotypes. Studies of the percent Hex A in the samples from Hex A pseudodeficient subjects containing the C739T mutation revealed very low levels in serum (0-15%), but higher levels in leucocytes (13-24%) and fibroblasts (8-26%) using the synthetic substrate, 4-MUG (Kelly *et al.*, 1976; O'Brien *et al.*, 1978; Vidgoff *et al.*, 1973; Thomas *et al.*, 1982; Grebner *et al.*, 1986; Navon *et al.*, 1986). When 4-MUGS was used as the substrate, samples from the C739T-mutation containing Hex A pseudodeficient subjects also showed lower serum Hex A activity than that in the leucocytes (Triggs-Raine *et al.*, 1992). However, the percent Hex A in serum (~13%) from the C745T-mutation containing subject showed a similar level to that in leucocytes (~16%) (Cao *et al.*, 1993).

In pseudodeficient fibroblasts, loading studies were done using an *in situ* assay. The $G_{M2}$ ganglioside that was not hydrolyzed accounted for 39-48% of the total-loaded $G_{M2}$ ganglioside (normal range = 39-49%, Tay-Sachs disease range = 89-92%) (Grebner *et al.*, 1986; Thomas *et al.*, 1982). Using $G_{M2}$ ganglioside as the substrate in an *in vitro* assay, in the presence of sodium taurocholate, the hydrolytic activities were in the range of 35-57% of normal (Kelly *et al.*, 1976; O'Brien *et al.*, 1977 and 1978; Thomas *et al.*, 1982; ).

These findings suggested that the benign mutations might reduce Hex A's capacity to hydrolyse synthetic substrates, but not its ability to hydrolyse $G_{M2}$ ganglioside as measured using the *in situ* and *in vitro* assay, as previously proposed by Triggs-Raine *et al.* (1992).

However, these *in vitro* assays for the hydrolysis of $G_{M2}$ ganglioside were done in the presence of detergent, and therefore obtained activities might include some Hex B activity. The *in situ* $G_{M2}$ ganglioside loading assay could not quantitatively measure the activity above the threshold for normal $G_{M2}$ ganglioside hydrolysis. In contrast, using an *in vitro* assay in the presence of $G_{M2}$ activator protein, but without detergent in the reaction, the residual Hex A activity in the pseudodeficient fibroblasts was determined to be about 11-20% of the normal activity toward $G_{M2}$ ganglioside (Conzelmann *et al.*, 1983). This was higher than the activities in the fibroblasts from adult-onset (2-4%), late-infantile (~0.5%) and infantile (~0.1%) forms of $G_{M2}$ gangliosidosis patients (Conzelmann *et al.*, 1983).

Analysis using polyacrylamide gel electrophoresis revealed an absence of Hex A activity in the serum and leucocytes of samples from Hex A pseudodeficient subjects (Kelly *et al.*, 1976; Vidgoff *et al.*, 1973; Thomas *et al.*, 1982). A reduced, but substantial, amount of Hex A was detected in the fibroblasts (Thomas *et al.*, 1982). Grebner *et al.* (1986) hypothesized that the lack of Hex A activity in serum might be due to (1) no secretion, (2) higher clearance rate in the blood circulation, or (3) increased lability. To determine the basis of serum Hex A deficiency, the tissue from which the secreted ß-hexosaminidase originated needs to be investigated.

Further analysis of Hex A containing the benign mutations showed an optimal pH value of 4.3-4.4 for maximal Hex A activity in the plasma, the same value as the normal control (Vidgoff *et al.*, 1973; Grebner *et al.*, 1986). A heat treatment of fibroblast samples at 37°C for 2 hr did not resulted in a decrease in Hex A activity similar to the normal control (Grebner *et al.*, 1986). Metabolic labelling studies in fibroblasts showed that the α-subunit

protein was processed to its mature lysosomal form (Thomas *et al.*, 1982; Grebner *et al.*, 1986). Although this analysis was not quantitative, the protein level was consistent with that which might be expected in a Tay-Sachs disease heterozygote (Thomas *et al.*, 1982) or slightly lower than that in the heterozygote (Grebner *et al.*, 1986).

The molecular analysis of the *HEXA* gene in individuals with Hex A pseudodeficiency has allowed the identification of benign mutations. Currently, two benign mutations, C739T(R247W) and C745T(R249W) (Triggs-Raine *et al.*, 1992; Cao *et al.*, 1993), and two putative benign mutations G746A (R249Q) (Callahan *et al.*, 1995) and G748A(G250S) (Triggs-Raine *et al.*, 1995) have been reported. All the identified Hex A pseudodeficient subjects, but one, were found to be compound heterozygotes having one of the known benign mutations on one chromosome and a common Tay-Sachs disease mutation on the other (Triggs-Raine *et al.*, 1992; Cao *et al.*, 1993).

The first benign mutation, C739T(R247W), was found in compound heterozygosity with either the 4-bp insertion or IVS-12 G→C Tay-Sachs disease-causing mutation on the second allele (Triggs-Raine *et al.*, 1992). Most identified Hex A pseudodeficient subjects have the C739T mutation (Triggs-Raine *et al.*, 1992; Grebner *et al.*, 1986; Bayleran *et al.*, 1986; Navon *et al.*, 1986; Thomas *et al.*, 1982). The frequency of this benign mutation was estimated to be 32% in non-Jewish and 2% in Jewish enzyme-defined carriers (Triggs-Raine *et al.*, 1992; Kaback *et al.*, 1993; Tomczak *et al.*, 1993).

The second benign mutation, C745T(R249W), was found in a healthy 38-year-old non-Jewish woman (Cao *et al.*, 1993). She was identified in a carrier screening program at the time her Ashkenazi Jewish spouse was being tested. Her Hex A activities toward 4-MUG

in serum and leucocytes were found to be in the Tay-Sachs disease range. Using 4-MUGS as the substrate, Hex A activity was 10 times higher than for Tay-Sachs disease in serum (40% of the normal), and 5 times higher than for Tay-Sachs disease in leucocytes (21% of the normal) (Cao *et al.*, 1993). Hex A activities of her parents were in the carrier range using both 4-MUG and 4-MUGS as the substrates. DNA analysis revealed the adult-onset mutation G805A(G269S) in the mother and the mutation C745T(R249W) in the father.

Two putative benign mutations, G746A(R249Q) and G748A(G250S), have been identified, but not been confirmed. The putative benign mutation, G746A(R249Q), was recently identified in a fetus, by analyzing a chorionic villus sample. The sample was found to contain the 4-bp insertion mutation on the second allele and exhibited 10-18% Hex A activity (Callahan *et al.*, 1995). The 4 bp insertion mutation was found in the mother and the R249Q mutation was found in the father who had 47% serum Hex A activity. In addition, a second putative benign mutation, G748A(G250S), was identified in 4 out of 36 enzyme-defined carriers in the Franco-American population (Triggs-Raine *et al.*, 1995). They are called putative benign because the G746A mutation was identified in a fetus and the G748A mutation was found only in carriers; neither have been identified in Tay-Sachs disease patients or normal (i.e. non-carrier) individuals. Expression studies on the G746A(R249Q) and G748A(G250S) mutations are required to characterize and confirm their identity as benign mutations.

How the benign mutations affect Hex A was unknown. Some investigators proposed that the enzyme's capacity to hydrolyse the synthetic, but not the natural substrate, was reduced (Kelly *et al.*, 1976; O'Brien *et al.*, 1978; Triggs-Raine *et al.*, 1992). Others

suggested that there was a reduction in the activity of the enzyme toward both synthetic and natural substrates, and that a differential tissue distribution accounted for the very low serum levels (Thomas *et al.*, 1982; Grebner *et al.*, 1986).

1.16 Mutations in exon 7 of the α-subunit.

Currently, seven mutations have been found in the exon 7 region of the α-subunit. These include the two benign mutations, C739T(R247W) and C745T(R249W), a juvenile-onset mutation G749A(G250D), two putative benign mutation G746A(R249Q) and G748A(G250S), a B1 mutation G772C(D258H) and an adult-onset mutation G805A(G269S). These are highly conserved residues in human and mouse *HEXA* and *HEXB*, pig *HEXB* and the ß-hexosaminidase in yeast, bacteria and fungi (Fig. 1). Based on a predicted structural model for chitobiase (Tews *et al.*, 1996), three of these residues R247, R249 and G269, are located on the surface of the α-subunit suggesting that they may not be involved in the catalytic reaction but might be involved in maintaining the stability of the α-subunit and/or interactions with the ß-subunit. The residue G250 falls in the same region of the α-helix and in proximity to the residues R247 and R249, but G250 faces inward. The residue D258 is located in close proximity to R178 in the catalytic pocket. In general, exon 7 may contain residues that have functions in maintaining the stability of the α-subunit and others that have a role in the catalytic reaction, depending on their placement in the tertiary structure of the enzyme.

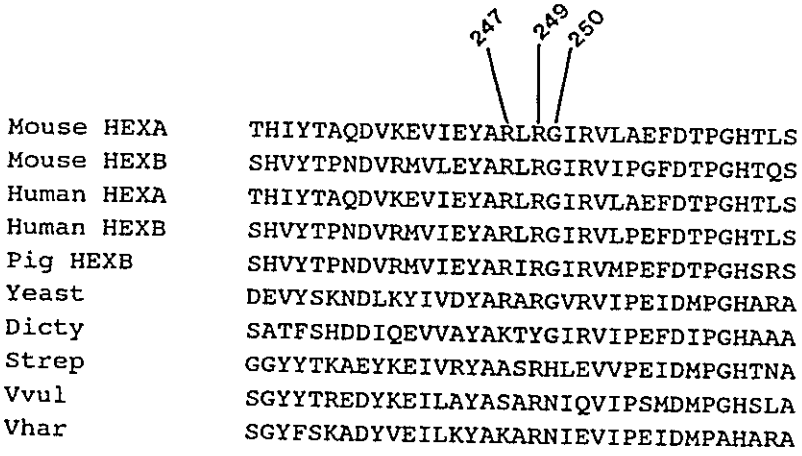



Fig.1. Homology between exon 7 region (amino acid sequence) of *HEXA* and other ß-hexosaminidases. Human HEXA and human HEXB indicate human α- and ß-subunits (Myerowitz *et al.*, 1985; O'Dowd *et al.*, 1985; Korneluk *et al.*, 1986); Mouse HEXA and mouse HEXB indicate mouse α- and ß-subunits (Bapat *et al.*, 1988; Beccari *et al.*, 1992); Pig HEXB indicates boar ß-subunit (unpublished, Genbank #X92379); Yeast indicates yeast ß-hexosaminidase (Cannon et al., 1994); "Dicty" indicates a region of ß-hexosaminidase from *Dictyostelium discoideum* (Graham *et al.*, 1988); "Strep" indicates a region of ß-hexosaminidase of *Streptomyces plicatus* (Triggs-Raine *et al.*, unpublished data); Vvul indicates a region of ß-hexosaminidase of *Vibrio vulnificans* (Somerville and Colwell, 1993); "Vhar" indicates a region of chitobiase of *Vibrio harveyi* (Soto-Gil and Zyskind, 1989). The positions of three amino acid residues, R247, R249 and G250, are indicated.

# 2. MATERIALS AND METHODS

2.0 Plasmid vectors.

2.0.0 pBluescript (pBS$^+$).

pBluescript (pBS$^+$), purchased from Stratagene (Aurora, ON), is a 2.96 kb vector used for routine DNA cloning experiments. It is derived from pUC19 and has the following features: (1) It contains a f1(+) filamentous phage origin of replication that allows the recovery of the sense DNA strand when a host strain is co-infected with helper phage. (2) It has a portion of the *lacZ* gene, encoding ß-galactosidase, that allows α-complementation to be used to differentiate recombinant from nonrecombinant plasmids by their white rather than blue colour in the presence of X-gal. (3) It carries an ampicillin-resistance gene for selection of plasmids in *E.coli.*

2.0.1 pRc/CMV-ß-gal.

pRc/CMV-ß-gal is a ß-galactosidase expression vector constructed by Zhilong Wang and provided by Dr. D. Litchfield (University of Western Ontario, London). It was transfected, together with other mammalian expression vectors, into Cos-7 cells as a control for the determination of transfection efficiency. The construct was prepared by subcloning a *Hind*III/*Sca*I DNA fragment containing *the lacZ* gene from pRSV-ß-gal (Promega, Madison, WI) into a pBS$^+$ vector digested with *Hind*III and *Sca*I to generate pBS-ß-gal. The DNA fragment containing *the lacZ* gene was released from pBS-ß-gal by digestion with *Hind*III and *Apa*II and subcloned into a pRc/CMV vector (Invitrogen, San Diego, CA), digested with *Hind*III and *Apa*II, to produce pRc/CMV-ß-gal.

2.0.2 pCD43 Vector.

The vector pCD43 was a gift from Dr. Roy Gravel (McGill University, Montreal). It contains a cDNA fragment encoding the ß-subunit of ß-hexosaminidase (O'Dowd *et al.*, 1985) and has previously been used for the expression of the ß-subunit in Cos-7 cells (Brown *et al.*, 1989; Trop *et al.*, 1992). The vector contains the SV40 early region promoter and two introns, normally used to splice late viral mRNAs, upstream of the multiple cloning region. There is an SV40 late region polyadenylation sequence downstream of the cloning site. This vector also possesses an ampicillin-resistance gene, a pBR322 origin of replication for propagation in *E. coli,* and an SV40 origin of replication that allows it to replicate in mammalian cells containing the SV40 large T antigen.

2.0.3 pSVL vector.

pSVL (Pharmacia Biotech, Baie D'Urfe, PQ) is an expression vector that can replicate in mammalian cells containing the SV40 large T antigen. It is 4896 bp long and contains both pBR322 and SV40 origins of replication. This vector also contains the SV40 late promoter and the SV40 VP1 processing signals for the transcription, splicing, and processing of a cDNA inserted in the multiple cloning region.

2.0.4 αpSVL.

The ß-hexosaminidase α-subunit cDNA was subcloned into the vector pSVL following a previously described strategy (Brown and Mahuran, 1993). Briefly, a 2 kb α-subunit cDNA was released from pTK18 (Myerowitz *et al.*, 1985) by digestion with *Nar*I/*Pst*I

and subcloned into pBS$^+$ digested with *Acc*I and *Pst*I to create pHHEXA2. The cDNA fragment from pHHEXA2 was released using *Xho*I and *Bam*HI and subcloned into pSVL cut by *Xho*I and *Bam*HI to create αpSVL. This construct was prepared by Dr. Barbara Triggs-Raine.

## 2.1 Determination of DNA and protein concentrations.

### 2.1.0 Spectrophotometric determination of DNA concentration.

This method was applied using the definition in "Molecular Cloning" by Sambrook *et al.* (1989) that "a solution containing 50 µg/ml of double-stranded DNA has an absorbance of 1 at 260 nm and a solution containing 40 µg/ml of single-stranded DNA has an absorbance of 1 at 260 nm". The DNA concentrations were calculated according to the following formulae.

For double-stranded DNA:

DNA concentration (µg/µl) = $A_{260\ nm}$ x 50 ÷ volume (µl) of added DNA sample

For single-stranded DNA:

DNA concentration (µg/µl) = $A_{260\ nm}$ x 40 ÷ volume (µl) of added DNA sample

### 2.1.1 Estimation of DNA concentration using DNA MASS™ ladder.

DNA MASS™ Ladder (Gibco/BRL) contained an equal molar mixture of six blunt-end DNA fragments from 100 to 2,000 bp. Electrophoretic separation of 4 µl of DNA MASS™ Ladder yielded bands containing 200, 120, 80, 40, 20 and 10 ng of DNA,

respectively. The DNA concentration of a sample was estimated by comparing the fluorescent intensity of DNA bands in the sample to bands in the DNA MASS™ Ladder after separation by electrophoresis on an 1% agarose gel containing ethidium bromide. Agarose gel electrophoresis will be described in section 2.2.

2.1.2 Determination of protein concentration.

Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad) based on the Bradford method (Bradford, 1976) and following the guidelines of the manufacturer. Bovine γ-globulin provided with the kit, diluted to provide 5 samples ranging in concentrations from 1.5 to 22.5 μg/ml, was used to generate a standard curve. The concentration of protein in a test sample was determined only using dilutions falling in the absorbance range of 0.15-0.30 at 595 nm.

2.2 Agarose gel electrophoresis.

Agarose gel eletrophoresis was used to separate DNA fragments based on size for analysis or subsequent manipulations.

Solutions:

5 x Tris-Borate Buffer (TBE): 0.45 M Tris base, 0.44 M boric acid, 1 mM EDTA, pH 8.0.

6 x Loading Buffer: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol.

Molecular Weight Marker (1 kb ladder): 30 μg (30 μl) of 1 kb ladder DNA (Gibco/BRL), 90 μl 6 x Loading Buffer, 180 μl of $ddH_2O$. The final concentration of the

marker DNA was 100 ng/µl.

Agarose (Gibco/BRL) was mixed with 100 ml 1 x TBE to make 0.8% to 2.5% (w/v) agarose gels. The agarose was melted in a microwave oven (~2 min), cooled to about 60°C, and 5 µl of 10 mg/ml ethidium bromide (final concentration is 0.5 µg/ml) was added. This mixture was poured into a plastic tray positioned with a comb (0.5-1.0 mm thickness) to make a horizontal slab gel.

DNA samples (10 µl) for separation on agarose gels were mixed with 1.5 µl of 6 x Loading Buffer and separated by electrophoresis at a constant voltage (50-100 V) until the front dye migrated an appropriate distance through the gel. DNA bands were visualized and photographed on an ultraviolet light transilluminator.

2.3 Phenol/chloroform extraction and ethanol precipitation of DNA.

Phenol/chloroform extraction followed by ethanol precipitation was used to purify and concentrate DNA samples. The DNA sample was mixed with an equal volume of buffer-saturated phenol, pH 8.0 (Gibco/BRL). The water-soluble phase (upper phase) was separated from the lower phenol phase by centrifugation (14,000 x g) for 2 min and transferred into a fresh tube containing an equal volume of chloroform. After mixing, the aqueous phase was again separated by centrifugation and transferred to a clean tube. The chloroform extraction was repeated, and the resulting aqueous phase was mixed with a 2.5 volumes of 95% ethanol and 0.1 volume of 3 M sodium acetate, pH 4.8. The mixture was incubated on ice for 15 min and the DNA was precipitated by centrifugation at 14,000 x g for 15 to 30 min. To remove excess salt, the DNA pellet was washed in cold 70% ethanol and precipitated by

centrifugation at 14,000 x g for 15 min. The DNA pellet was air-dried and suspended in sterile dd$H_2O$.

2.4 Bacterial culture.

Bacteria were used to amplify normal or mutant plasmid DNA and to generate single-stranded DNA.

2.4.0 Bacterial strains.

*E.coli* DH5α (Gibco/BRL) was used for the amplification of plasmid DNA. Genotype: ϕ80d*lacZ*ΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17 ($r_k^-$, $m_k^+$), *supE*44, *relA*1, *deoR*, Δ(*lacZYA-argF*)U169.

*E.coli* BMH71-18 (Promega, Madison, WI) was used to amplify plasmid DNA harbouring nucleotide base mismatches. This was done as a part of the Unique Site Elimination (USE) method (Deng and Nickoloff, 1992). Genotype: *thi*, *supE*, *Δ*(*lac-proAB*), [*mutS*::Tn10] [F', *proAB*, *laq*$I^q$*Z*ΔM15].

*E. coli* JM109 (Promega, Madison, WI) was used for the preparation of single-stranded DNA. Genotype: *recA1*, *supE44*, *endA1*, *hsdR17*, *gyrA96*, *relA1*, *thi*, *Δ*(*lac-proAB*), F'[*traD36*, *proAB*$^+$, *laq*$I^q$*ZΔM15*].

2.4.1 Medium for bacteria culture.

LB Media. To prepare LB liquid medium, 25 g of Miller's LB Broth Base (Gibco/BRL) was dissolved in 1 liter of distilled water and autoclaved before use. To prepare

LB agar plates, 10 g of Bacto-agar (Difco, Detroit, Michigan) was added to the medium before autoclaving; the medium was cooled to approximately 50°C and poured into petri dishes. To prepare LBA-agar or liquid medium, ampicillin (50-100 μg/ml) was added after the medium had cooled below 50°C. LBT-agar or liquid medium was made by adding tetracycline (~10-30 μg/ml) to the medium after it had cooled.

M9 Minimal Media. To prepare M9 minimal medium, a solution containing 42 mM $Na_2HPO_4$, 22 mM $KH_2PO_4$, 8.5 mM NaCl and 19 mM $NH_4Cl$, pH 7.4, was autoclaved, cooled and supplemented with pre-sterilized solutions of 1 M $MgSO_4$ (2 ml), 1 M $CaCl_2$ (0.1 ml), and 20% glucose (10 ml). For *E.coli* strains that were *thi*, thiamine-HCl (1 mM) was also added to the cooled medium. To make M9-agar plates, Bacto-agar (10 g/litre) was added to the M9 minimal medium just before autoclaving; the medium was cooled to approximately 50°C and poured into petri dishes.

Low-Salt LB Medium. This medium was prepared by dissolving 5 g bacto tryptone, 2.5 g bacto yeast extract and 2.5 g NaCl in 500 ml of $ddH_2O$. The pH was adjusted to 7.0 and the medium was autoclaved.

SOC Medium. To prepare this medium, bacto-tryptone (20 g), bacto yeast extract (5 g) and NaCl (0.5 g) were dissolved in 985 ml of $ddH_2O$. KCl (0.19 g) was added and the pH was adjusted to 7.0 with NaOH. After the solution was autoclaved and cooled below 60°C, 5 ml 2 M $MgCl_2$ and 10 ml 2 M glucose, both sterilized by filtration, were added. SOC medium was stored at -20°C for subsequent use.

2.4.2 Storage of Bacteria in glycerol.

Sterile glycerol (0.15 ml) was mixed with 0.6-0.8 ml of an overnight bacterial culture and this mixture was stored at -20°C. To recover the bacteria, a sterile inoculating loop was used to remove bacteria from the storage tube and this was immediately streaked onto the surface of an LB-agar plate containing antibiotics, as required, for plasmid or episome selection.

2.4.3 Bacterial subculture.

Bacterial cultures were inoculated from a single colony on a LB-agar plate, with or without antibiotics, into 10 ml of liquid medium and grown overnight at 37°C with vigorous shaking (350 rpm). This overnight culture was used as an inoculum (1/100 dilution) to prepare fresh log-phase or large volume cultures.

2.5 Bacterial transformation.

Bacterial transformation was done to introduce plasmid DNA into bacteria by electroporation, following the protocols provided by the manufacturer of the ELECTRO CELL MANIPULATOR 600 (San Diego, CA).

2.5.0 Preparation of competent cells.

Bacterial cells were grown in LB broth (low-salt) at 37°C to a density of approximately $10^{10}$ cells/ml (0.5-1.0 $OD_{600}$). The cells were pelleted by centrifugation at 4,000 x g for 15 min at 4°C and washed four times as outlined below. The cell pellet was washed once by resuspension in 1 volume of sterile cold dd$H_2O$ followed by centrifugation

at 4,000 x g for 15 min at 4°C. For washes 2 and 3, the cell pellet was resuspended in 0.5 volume sterile cold dd$H_2O$ and pelleted by centrifugation at 4,000 x g for 15 min at 4°C. For wash 4, the cell pellet was resuspended in 0.02 volume of sterile cold dd$H_2O$ and again pelleted. After the final wash, the cells were resuspended in 0.002-0.003 volume of sterile-filtered cold 10% glycerol; aliquots were stored at -80°C and thawed on ice for 5-10 min before use.

2.5.1 Electroporation for transformation of plasmid DNA into *E. coli*.

The electro-competent bacterial cells (40 µl) were mixed with 1 µl (~1 ng) of plasmid DNA prepared in sterile dd$H_2O$ or diluted 5-fold from a ligation. The mixture was transferred to an ice-cold sterile 1 mm gap cuvette (BTX Inc., San Diego) and electroporated using an ELECTRO CELL MANIPULATOR 600 (BTX Inc., San Diego). The settings for electroporation were recommended by the manufacturer (2.5 kV/resistance high voltage (HV), 129 ohm, 1.3-1.5 kV/Set charging voltage). After the electroporation, 960 µl of SOC medium was added, the mixture was transferred into a fresh tube, and incubated at 37°C for 1 hr with shaking. One hundred µl of the suspension, after incubation, was evenly spread on a LBA-agar plate and incubated at 37°C overnight.

2.6 Plasmid DNA isolation.

Plasmid DNA was isolated from *E.coli* in large-scale (more than 100 ml culture) using a Nucleobond-AX column (The Nest Group Inc. Southbora, MA) and following the protocol provided by the manufacturer. The protocol was primarily based on the alkaline/SDS

extraction procedure of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981). A modification of this protocol was used for small-scale (less than 2 ml culture) plasmid DNA preparation.

Solutions:

S1: 50 mM Tris-HCl, 10 mM EDTA, 100 µg RNase A/ml, pH 8.0

S2: 200 mM NaOH, 1% SDS

S3: 2.60 M KAc, pH 5.2

N2: 100 mM Tris, 15% ethanol, 0.9 M KCl, pH adjusted to 6.3 with $H_3PO_4$

N3: 100 mM Tris, 15% ethanol, 1.15 M KCl, pH adjusted to 6.3 with $H_3PO_4$

N5: 100 mM Tris, 15% ethanol, 1 M KCl, pH adjusted to 8.5 with $H_3PO_4$

2.6.0 Large scale plasmid DNA preparation.

A bacterial cell pellet from about 125 ml of overnight culture was suspended in 12 ml of buffer S1 and then gently mixed with 12 ml of buffer S2. The mixture was allowed to incubate at room temperature for 5 min and then mixed with 12 ml of buffer S3 by gently inverting the tube (7-8 times) until a homogeneous suspension was formed. After a 5 min incubation on ice, the cell debris were pelleted by centrifugation at 14,000 x g for 20-30 min at 4°C. The resulting cleared supernatant was loaded on a cartridge (AX 500) that was previously equilibrated with 5 ml of buffer N2. The cartridge was then washed twice with 12 ml of buffer N3 and plasmid DNA was eluted into a fresh tube with 6 ml of buffer N5. The eluent was mixed with 0.7 volume (4.2 ml) of isopropanol, followed by centrifugation at 14,000 x g at 4°C for 20-30 min to precipitate the purified plasmid DNA. The resulting DNA

pellet was washed once with 70% ethanol, air-dried (about 5 min) and dissolved in sterile $ddH_2O$. The amount of sterile $ddH_2O$ was varied depending on the size of the DNA pellet.

2.6.1 Small-scale plasmid DNA preparation.

The initial steps for cell lysis and production of a cleared supernatant were the same as those described for large-scale plasmid DNA preparation except that: (1) 1.5 ml of an overnight culture was used, (2) the volume of S1, S2 and S3 buffer was reduced to 200 µl, and (3) centrifugation was performed at 14,000 x g in a microcentrifuge. After high-salt (S3) precipitation, the cleared supernatant was mixed with 0.6 ml of 95% ethanol, incubated on ice for 15 min, and centrifuged at 14,000 x g at 4°C for 10 min to obtain a DNA pellet. The DNA pellet was washed once with 70% ethanol, air-dried and resuspended in 100 µl of sterile $ddH_2O$.

The DNA prepared using this protocol was used for all recombinant DNA procedures except sequencing. For DNA sequencing, the DNA preparation was extracted with phenol/chloroform and precipitated with ethanol.

2.7 Isolation of DNA bands from agarose gels using a GENECLEAN II kit.

DNA fragments were purified from agarose gels using a GENECLEAN II kit (BIO 101, Inc. Vista, CA) and following the guidelines of the manufacturer. The DNA band of interest was excised from an agarose gel, after separation by electrophoresis, and placed in an 1.5 ml microfuge tube. The weight of the agarose gel was determined to predict the volume of the gel. The gel was melted in 0.5 volume of TBE modifier and 4.5 volumes of

sodium iodide (supplied with the kit) at 55°C for approximately 5 min. The resulting melted agarose/iodide mixture was thoroughly mixed with 5 µl of GLASSMILK™ (specially formulated silica matrix supplied with the kit) and incubated on ice for 5 min. The GLASSMILK, which binds DNA (Vogelstein and Gillespie, 1979), was pelleted by centrifugation for 20 second at 14,000 x g. The pellet was washed with 1 ml of NEW WASH solution (supplied with the kit and kept at -20°C); this step was repeated two additional times. After the final wash, a short centrifugation step was used to move the residual NEW WASH solution to the bottom of the tube for removal. The pellet was suspended in 10 µl of sterile $ddH_2O$ and incubated at 55°C for 5 min to release the DNA from the GLASSMILK. The GLASSMILK was then removed by centrifugation at 14,000 x g for 1 min and the DNA-containing supernatant was transferred to a clean tube.

2.8 Sources and preparation of genomic DNA.

Leucocyte pellets or sonicates from enzyme-defined carriers, obligate carriers and non-carriers of Ashkenazi Jewish (descended from Jews from central or eastern Europe) and non-Jewish ancestry, who were identified by carrier screening through the California and Philadelphia Tay-Sachs Disease Prevention Programs, were provided for analysis for the C745T mutation. Leucocyte pellets from the proband (16819) and her parents were provided by the Boston Tay-Sachs Disease Prevention Program.

Genomic DNA for analysis was prepared from leucocyte pellets or sonicated leucocyte pellets using a modified version of the procedure of Hoar *et al.* (1984). Briefly, a portion of each leucocyte pellet resuspended in 100 µl $ddH_2O$, or 100 µl of sonicated

leucocyte pellet, was mixed with 2 µl of 10% SDS. The mixture was incubated at room temperature for 5 min and then phenol-extracted as described above. The phenol phase was re-extracted with an equal volume of water and the aqueous phases were combined for a chloroform extraction as described above. After ethanol precipitation, the resulting DNA pellet was air-dried and suspended in 100 µl $ddH_2O$. Sterile reagents and solutions were used throughout this procedure.

2.9 Restriction enzyme digestion.

Restriction enzyme digestions of plasmid DNA or PCR-products were done to determine the identity of DNA fragments or to detect mutations. A restriction enzyme digest of plasmid DNA contained 0.1 - 0.5 µg DNA, 1 µl of 10 x restriction enzyme buffer (provided by the enzyme manufacturer) and 1 - 2 U restriction enzyme (NEB, Mississauga, ON or Gibco/BRL, Burlington, ON) in a total volume of 10 µl.

A restriction enzyme digest of PCR-product contained 10 µl (~ 0.1-0.3 µg DNA) of PCR product in the PCR reaction mix and 1 - 2 U restriction enzyme. The buffer provided with the restriction enzyme was not used, unless indicated.

Reactions were incubated at the temperature recommended by the manufacturer for 1-2 hr. The sample (10 µl) then was mixed with 1.5 µl 6 x Loading Buffer and analyzed directly on an agarose gel (see section 2.2). Alternatively, the reaction was stopped by adding 0.5 M EDTA (pH 8.0) to a final concentration of 10 mM or by freezing the sample at -20°C for further analysis.

2.10 Polymerase chain reaction (PCR).

The polymerase chain reaction (Saiki *et al.*, 1985) was used to amplify specific regions of genomic or plasmid DNA. Each reaction contained the following components: 80 µl $ddH_2O$, 10 µl 10 x PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$ and 0.1% gelatin), 2 µl each 10 mM dNTP (ie. dATP, dCTP, dGTP and dTTP from Pharmacia), 1 µl sense primer (100 ng/µl), 1 µl anti-sense primer (100 ng/µl), 0.3 U Taq DNA polymerase (Gibco/BRL), and 0.2 to 0.5 µg genomic DNA or 5 to 50 ng plasmid DNA or a portion of a plasmid-transformed bacterial colony.

The reactions were overlaid with approximately 50 µl of paraffin oil and the PCR reaction was performed for 32 cycles in a Perkin Elmer Cetus DNA Thermal Cycler. One cycle included 30 sec of denaturation at 94°C, 30 sec of annealing at 60°C, and 90 sec of extension at 72°C. Sterile or dedicated solutions and equipment were used for this procedure.

2.11 Double-stranded DNA sequencing.

Double-stranded DNA was sequenced using the dideoxynucleotide chain termination method first described by Sanger *et al.* (1977).

2.11.0 Alkali denaturation of double-stranded (ds)DNA.

For each sequencing reaction, 2 µg dsDNA template was denatured by incubating with 8 µl 2 M NaOH in a total volume of 40 µl for 10 min at room temperature. The denatured DNA was precipitated by mixing with 7 µl 3 M sodium acetate, pH 4.8, 4 µl sterile

$ddH_2O$ and 120 µl 95% ethanol, followed by centrifugation at 14,000 x g for 10 min. The DNA pellet was washed once with 70% ethanol, air-dried and dissolved in 10 µl sterile $ddH_2O$.

2.11.1 Annealing primer to the DNA template.

The denatured DNA was annealed with the oligonucleotide sequencing primer by mixing 2 µl (5-10 pmol or 15-30 ng) primer, 10 µl (2 µg) denatured DNA and 2 µl annealing buffer provided with T7 sequencing ™ Kit (Pharmacia Biotech, Baie D'Urfe, PQ). This mixture was incubated at 65°C for 5 min, 37°C for 10 min and room temperature for 5 min.

2.11.2 Labelling reaction, extension and separation.

The primer, now annealed with the DNA template, was extended at room temperature for 5 min in a mixture containing 14 µl template/primer mix, 3 µl labelling mix (provided with T7 sequencing ™ Kit), 1 µl [$\alpha$-$^{35}$S]dATP (1,000 Ci/mmol, NEN) and 2 µl diluted T7 DNA polymerase (provided with the T7 sequencing ™ Kit). T7 DNA polymerase was diluted 5 times with the dilution buffer provided with the T7 sequencing ™ Kit. The extension was randomly terminated by adding 4.5 µl of the reaction mix to prewarmed 2.5 µl aliquots of termination reaction mix of ddATP, ddCTP, ddGTP, or ddTTP (provided with the T7 sequencing ™ Kit). The random termination reaction was done at 37°C for 5 min followed by the addition of 5 µl of the stop solution provided with the T7 sequencing ™ Kit. The synthesized single-stranded DNA fragments were denatured at 100°C for 5 min and separated at 60 W for 2-4 hr on a 6% polyacrylamide (Gibco/BRL) gel containing 42% urea

(Gibco/BRL).

2.12 Site-directed mutagenesis.

Three methods of site-directed mutagenesis were used to introduce mutations into the α-subunit cDNA in αpSVL to produce mutant αpSVLs.

2.12.0 Introduction of the C739T (R247W) mutation into αpSVL.

The C739T(R247W) mutation was introduced into αpSVL by replacing a *Nde*I/*Sna*BI fragment with the same fragment from isolated cDNA clone containing the C739T(R247W) mutation. This construct was made and provided by Dr. B. Triggs-Raine.

2.12.1 A modified Unique-Site-Elimination (USE) mutagenesis method.

The USE method (Deng and Nickoloff, 1992) was modified, as outlined below, and used to introduce the C745T(R249W) substitution into αpSVL. In principle, two mutant oligonucleotide primers, one containing the desired mutation and the other containing a mutation that destroyed a restriction enzyme site in the vector, were annealed with denatured αpSVL and extended to synthesize the second DNA strand. After amplification in an *E. coli* strain that does not repair mismatches, mutation-containing colonies were identified with a PCR-based strategy that detected a specific mutation.

Primer Selection and Phosphorylation. Two primers were used in the USE method. The selection primer, WPG19 5'-TCGTCTTCA(T)GAAT(A)CTCATGTTT-3', containing two base changes (shown in parenthesis) introduced a new *BspH*1 site into the vector but

destroyed an existing *EcoR*I site. The mutagenic primer, WPG20 5'-TACGCACGGCTC(T)GGGGTATCCG-3', contained a C745T change (shown in parenthesis). Two separate reactions were conducted to phosphorylate the 5' OH group of each oligonucleotide. Each reaction (40 µl) contained 8 µl (8 µg) of one primer, 8 µl 1 mM ATP, 4 µl 10 x T4 polynucleotide kinase buffer (NEB, see the recipe below), 2 µl (~20 U) of T4 polynucleotide kinase (NEB) and 18 µl sterile $ddH_2O$. The 10 x T4 polynucleotide kinase buffer was comprised of 70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$ and 5 mM DTT. The reaction mixtures were incubated at 37°C for 2 hr and the phosphorylated primers were used in the next step.

Annealing and Extension of the Primers. To anneal the WPG19 and WPG20 primers to the template, 4 µl (0.4 µg) of αpSVL DNA was mixed with 20 µl of phosphorylation reaction mixtures containing 4 µg of the each primer, and was denatured at 100°C for 5 min, then immediately placed on ice. For extension, the following components were added: 44 µl sterile $ddH_2O$, 2 µl (17 µg) bovine serum albumin (NEB), 4 µl each of 5 mM dATP, dCTP, dGTP and dTTP, 11 µl 10 x T4 DNA polymerase buffer (NEB), and 6 µl (18 U) T4 DNA polymerase (NEB). The reaction mix (101 µl) was incubated at 37°C for 3 hr, then 10 µl 10 x ligase buffer (NEB) and 1 µl (2,000 U) T4 DNA ligase (NEB) were added. The reaction was continued at room temperature overnight, phenol/chloroform extracted and ethanol precipitated (see section 2.3). The resulting DNA was suspended in 15 µl of sterile $ddH_2O$. The selection primer was used in the reaction to synthesize the second DNA strand. However, it was not used for selection because digestion with *EcoR*I, to differentiate parental DNA from the newly synthesized DNA, resulted in complete digestion indicating that the

oligonucleotide was poorly incorporated.

Transformation into *E. coli* for Amplification. The DNA ligation product was diluted 5 fold with sterile dd$H_2O$ and 1 µl was transformed into electro-competent *E.coli BMH* 71-18 *mut S* (see section 2.5). One hundred µl of the transformed bacteria were spread on an LBA-agar plate and incubated at 37°C overnight. Single colonies were streaked on a fresh LBA-agar plate and incubated at 37°C overnight. A portion of each subcultured colony was used as a source of DNA for PCR to screen for the C745T change (see section 3.1.1). A C745T positive colony was identified, cultured in 10 ml LBA medium and plasmid DNA was prepared using the small-scale plasmid DNA preparation method (see section 2.6.1). The isolated C745TαpSVL plasmid DNA was then transformed into *E.coli* DH5α. The transformed *E.coli* DH5α cells were grown on a LBA-agar plate and colonies were screened for the presence of αpSVL with the C745T change. The positive colonies were identified, amplified and stored in glycerol at -20°C; one of the positive colonies was used to prepare the plasmid in large scale.

2.12.2 *In vitro* oligonucleotide-directed mutagenesis.

A procedure based on that of Kunkel *et al.* (1987), but using T7 polymerase (Bebenek and Kunkel, 1989), was used to introduce the mutations, G805A(G269S), G749A(G250D), and C508T(R170W) into pHHEXA2. The cDNA fragment containing the mutation was then released from pHHEXA2 and subcloned into pSVL to generate the corresponding mutant αpSVL vectors.

Preparation of Single-Stranded DNA (ssDNA). The ssDNA of pHHEXA2 was

prepared according to a procedure by Vieira and Messing (1987). The plasmid pHHEXA2 was transformed into JM109 and the cells were grown at 37°C in LB medium. A culture of JM109/pHHEXA2, in early-log phase, was infected with 1 x $10^8$ pfu/ml of M13K07 helper phage (NEB) and incubated at 37°C for 75 min with shaking at 150 rpm. Kanamycin (70 µg/ml) was added and the incubation at 37°C was continued for 18 hr. The cells were pelleted by centrifugation at 4,000 x g for 10 min, the supernatant was transferred into a fresh tube, and one ninth volume of 40% polyethylene glycol (Sigma) and of 5 M sodium acetate was added. The mixture was incubated on ice for 30 min and the phage was pelleted by centrifugation at 8,000 x g for 10 min. The pellet was suspended in 200 µl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) and the DNA was purified from the phage by phenol/chloroform extraction and ethanol precipitation (see section 2.3).

Annealing of the Primer to the ssDNA. Primers containing the desired nucleotide substitution were annealed with the pHHEXA2 ssDNA. Each reaction contained 1 µl (0.1 pmol) of pHHEXA2 ssDNA, 1 µl (~0.2 pmol) of the mutagenic oligonucleotide, 1 µl of 10 x annealing buffer (20 mM Tris-HCl, pH 7.4, 2 mM MgCl, 50 mM NaCl), and sterile dd$H_2O$ to a total volume of 10 µl. The mutagenic oligonucleotides 5'-TCCTGGGGACCAAGTATCCCTGGA-3', 5'-ACGGCTCCGGGATATCCGTGTGC-3', and 5'-GCTTTCCTCACTGGGGCTTGCTG-3' were 5' end phosphorylated before they were purchased (University DNA Core Facility, Calgary). These were used in three separate reaction mixes to create the substitutions G805A(G269S) (Navon and Proia, 1989; Paw *et al.*, 1989), G749A(G250D) (Trop *et al.*, 1992), and C508T(R170W) (Fernandes *et al.*, 1992b), respectively. Annealing was done by incubating the reaction mixture for 60 min in

an 1 litre beaker containing more than 0.5 litre of 70$^0$C water that was placed on ice.

Synthesis of the Complementary DNA Strand. To extend the annealed primer, and ligate the ends, the following components were added to the reaction mix while it was still in the water bath: 1 µl 10 x synthesis buffer (0.4 mM each dNTP, 0.75 mM ATP, 17.5 mM Tris-HCl, pH 7.4, 3.75 mM $MgCl_2$, and 21.5 mM DTT), 1 µl (3 U) T4 DNA ligase (Gibco/BRL), and 1 µl (0.5 U) T7 DNA polymerase (Form II, unmodified, NEB) diluted with dilution buffer (20 mM potassium phosphate, pH 7.4, 1 mM DTT, 0.1 mM EDTA, 50% glycerol) that was provided by the manufacturer. After a 5 min incubation on ice, the incubation was continued at room temperature for 5 min, and at 37$^0$C for 30 min. The whole reaction mix (14 µl) was then immediately analyzed by agarose (1%) gel electrophoresis. Double-stranded and single-stranded pHHEXA2 DNA samples were also separated by electrophoresis as controls.

Isolation and Identification of Mutant pHHEXA2-Containing Colonies. After agarose gel electrophoresis, the DNA band in the reaction sample that appeared similar in size to that of the control dsDNA was excised from the gel. The DNA was purified using a GENECLEAN II kit (see section 2.7) and 20-40% of the GENECLEAN product was transformed into *E coli*/DH5α. The colonies that contained the mutant vector were identified by PCR of the relevant region, followed by restriction enzyme digestion with an enzyme whose site was altered by the mutation. One colony was cultured for small-scale plasmid preparation. The mutation-containing α-subunit cDNA fragment was released from pHHEXA2 by digestion with *Bam*HI and *Xho*I, and subcloned into pSVL cut with *Bam*HI and *Xho*I to create the various mutant αpSVL(s). In all cases, the entire cDNA insert was sequenced to confirm the presence of the desired, but no additional, base changes.

2.13 Mammalian cell culture.

Two types of mammalian cell lines were used, Cos-7 and fibroblast. Cos-7 cells were used as a host for the transient expression of normal and mutant ß-hexosaminidase. Normal and benign mutation-containing fibroblasts were used to confirm and extend the observations obtained from the analyses in Cos-7 cells.

2.13.0 Cell lines.

Cos-7. Cos-7, obtained from the American Type Culture Collection (Rockville, MD), is a monkey kidney fibroblast-like cell line. It was established by Gluzman (1981) by transforming a replication origin-defective SV40 into CV-1 simian cells. The resulting Cos cells retained complete permissiveness for the growth and replication of SV40 and produced wild type T antigen. The Cos cells allow SV40, containing a DNA insert in the early region, to be replicated. Thus, it is a good host for the transient expression of transfected DNA in the SV40-based vector pSVL.

Hex A Pseudodeficient Fibroblast Cell Lines. TC72 (Thomas *et al.*, 1982) and GM04863 (Grebner *et al.*, 1986) are skin fibroblast cell lines established from forearm biopsies from individuals with Hex A pseudodeficiency. They were obtained from Dr. George Thomas (The Kennedy Institute, Baltimore, MD) and the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ), respectively. These samples were identified as subjects B (GM04863) and F (TC72) in a previous study (Triggs-Raine *et al.*, 1992) where they were shown to be compound heterozygotes for the C739T(R247W) benign mutation and a second mutation associated with Tay-Sachs disease. The second allele in GM04863 was 1278ins4

(Myerowitz and Costigan, 1988) and in TC72 it was 1073+1G→A (Akerman *et al.*, 1992; Mules *et al.*, 1992).

Control Fibroblast Cell Lines. The normal fibroblast line WP09 was from the University of Manitoba (Winnipeg). The normal cell line MCH065 and the Tay-Sachs disease cell line WG1881 were from The Repository for Mutant Human Cell Strains, Montreal, PQ. The Tay-Sachs disease line 1492 was from Hospital for Sick Children (Toronto). The Sandhoff disease cell line GM00294 was from NIGMS Human Genetic Mutant Cell Repository, Camden, NJ.

2.13.1 Cell culture, subculture and cell storage.

Cell Culture. Cos-7 and human fibroblast cell lines were grown on tissue culture dishes containing α-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum and penicillin (100 U/ml)/streptomycin (100 µg/ml) at 37°C in 5% $CO_2$.

Subculture. Cell lines were subcultured when they reached near-confluence (80-90%). Briefly, the medium in the culture dish was removed by aspiration and the cell monolayer was rinsed once with 2-3 ml of trypsin/EDTA solution (0.25% trypsin, 1 mM EDTA, from Gibco/BRL). Two ml of trypsin/EDTA were added and the dish was incubated at room temperature for 3-5 min. The dish was tapped lightly to detach adhered cells, 8 ml of α-MEM was mixed with the cell suspension and 1 ml of the mixture was dispensed into a fresh dish (20 x 100 mm or 20 x 150 mm) containing 15 ml or 30 ml of α-MEM supplemented with 10% fetal bovine serum and antibiotics.

Cell Storage. To store cells for long-term use, the cell suspension derived by

trypsinization, as described above, of a 20 x 150 mm dish was pelleted in a clinical centrifuge (IEC HN-SII Centrifuge, DAMON/IEC DIVISION) at 4,000 x g for 10 min. The pellet was resuspended in 1.5 ml of α-MEM supplemented with fetal bovine serum and antibiotics, and 1.5 ml of Cell Culture Freezing Medium-DMSO (Gibco/BRL). The cell suspension was mixed, dispensed into three cryovials, and kept at -80°C overnight. The cryovials were then transferred into liquid nitrogen for storage.

To recover stored cells, a cryovial was removed from liquid nitrogen and placed in a 37°C water bath until the cell suspension started to thaw. A small volume (0.5 ml) of prewarmed cell culture medium (37°C) was added to the cryovial. The mixture was quickly transferred into a tissue culture dish (20 x 150 mm) containing 30 ml of prewarmed culture medium and incubated at 37°C with 5% $CO_2$ for 24 hr. The medium was then replaced with fresh α-MEM.

2.14 Cos-7 cell transfection.

Cos-7 cells were used as a host for transfected DNA constructs designed to express normal and mutant Hex S and Hex A & S. DNA constructs were introduced into Cos-7 cells by electroporation.

Solutions:

Phosphate-buffered-saline (PBS) buffer: 1.1 mM $KH_2PO_4$, 8.1 mM $Na_2HPO_4$, 2.7 mM KCl, 138 mM NaCl, pH 7.6.

Isoton™II (Coulter Electronics Ltd., Florida): 136 mM NaCl, 1.3 mM disodium EDTA, 5 mM KCl, 1.4 mM $NaH_2PO_4$, 14 mM $Na_2HPO_4$, 7 mM NaF.

2.14.0 Cell counting.

To determine the number of Cos-7 cells prior to transfection, 60-80% confluent cells, collected from 6-12 dishes (20 x 150 mm) in trypsin/EDTA solution, were diluted to 50 ml in a sterile conical tube with sterile PBS buffer. A portion of the cell suspension was counted using a Coulter Particle Counter (Coulter Electronics Ltd., Florida). Briefly, a 0.5 ml aliquot of the diluted cell suspension was evenly suspended in a cell counting vial containing 19.5 ml of Isoton II solution using a syringe fitted with an 18 gauge needle. The Coulter Particle Counter's settings were: current-100, full scale-1, polarity-auto, attenuation-32, preset gain-4, lower threshold-3.0, upper threshold-99.9 and alarm limit-30. Three separate 0.5 ml counts were done and averaged. The total number of cells in 50 ml of PBS buffer was calculated by multiplying the average cell count by the dilution factor (4,000).

2.14.1 DNA transfection.

The Cos-7 cell suspension (12-36 x $10^6$ cells) was centrifuged at 4,000 x g for 10 min in a clinical centrifuge. The cell pellet was washed once in 50 ml of sterile PBS and resuspended in sterile PBS to give 8 x $10^6$ cells/ml. Each aliquot (360 µl) of cell suspension, containing approximately 2.9 x $10^6$ cells, was mixed with 40 µl of purified plasmid DNA (concentration varied) in a 1.5 ml centrifuge tube and the mixture was transferred into a pre-cooled sterile 2 mm gap electroporation cuvette (BTX Inc., San Diego). Electroporation was carried out using an ELECTRO CELL MANIPULATOR 600 (BTX Inc., San Diego) using the settings: 150 charging voltage, 48 ohms, and 1200 µF, which were based on a previous report (Chang *et al.*, 1991) and personal communication with Tim Salo of Dr. B. Triggs-

Raine's laboratory. This was followed by a 5-10 min incubation on ice. The electroporated sample was then transferred to a sterile tissue culture dish (20 x 150 mm) containing 25 ml of prewarmed α-MEM and incubated as described in section 2.13.1. At 24 hr post-transfection the medium was replaced. The cells were harvested at 72 hr post-transfection.

2.15 Preparation of cell and medium extracts.

Extracts were prepared from cultured cells and the media of cell cultures for further analysis.

2.15.0 Preparation of cell extract for protein and ß-hexosaminidase activity assays.

For the determination of protein concentration and enzyme activity levels, cell extracts of transfected Cos-7 cells and fibroblasts were prepared using the same procedure. The cell monolayer of a 20 x 100 mm or 20 x 150 mm dish was washed twice with ice-cold PBS and scraped off the plate into TEN buffer (40 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl). The cell suspension was centrifuged at 14,000 x g for 10 min and the cell pellet was resuspended in 20 mM Tris-HCl, pH 7.0. The cell extracts were prepared by three rounds of freezing (-80°C) and thawing (37°C) in 20 mM Tris-HCl, pH 7.0, followed by a centrifugation at 14,000 x g for 20 min. For some experiments using fibroblasts, 200 μM Leupeptin (acetyl-Leu-Leu-Argininal, from Sigma, Mississauga, ON) was added to the cell culture medium when the cells were about 60% confluent. The cells were harvested 48 h later, and cell extracts were prepared in the presence of 200 μM Leupeptin.

2.15.1 Preparation of medium and cell extracts for immunoprecipitation.

A protocol by Proia *et al.* (1984) was modified and used for the preparation of cell extracts.

Medium Extract. To prepare the medium extract, the medium that the cells had grown in was transferred from a tissue culture dish to a tube containing bovine serum albumin (1.5 mg/ml as a final concentration) and 4-(2-aminoethyl)-benzenesulfonyl fluoride (Pefabloc, from Boehringer Mannheim) to 1 mM as a final concentration. This was immediately centrifuged at 4,000 x g for 10 min to pellet suspended cells from the medium. The supernatant was saturated with $(NH_4)_2SO_4$ to 70% and centrifuged at 12,000 x g for 20 min. The resulting pellet was dissolved in 1 ml of TBS solution (10 mM Tris-HCl, pH 7.6, 150 mM NaCl) and dialysed overnight against 4 litres of the same buffer.

Cell Extract. To prepare the cell extract, the cell monolayer was washed twice with 5 ml of ice-cold PBS and the plates were kept on ice. Cells were scraped into 0.5 ml of lysis buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P40, 0.02% $NaN_3$, 1 mM Pefabloc) and evenly suspended by repeated pipetting. The cell suspension was incubated at 4°C for 10 min, with rocking, to lyse the cells. Insoluble cell debris were pelleted and the supernatant was collected as a cell extract. An aliquot (30 µl) of the cell extract was taken for protein determination. The remaining cell extract was immediately mixed with 50 µl of 100 mg/ml BSA solution and stored at -80°C.

2.16 ß-Hexosaminidase assay.

ß-Hexosaminidase activity was measured using 4-MUG as the substrate and according

to the protocol by Kaback (1977 b). Hex A & S activity was determined using 4-MUGS, the substrate specific to the α-subunit (Kresse *et al.*, 1981; Prence *et al.*, 1993).

Solutions:

Glycine-Carbonate Buffer: 0.17 M glycine, 0.17 M sodium carbonate, adjusted pH to 10.0 with NaOH.

Citrate-Phosphate Buffer: 6 mM Citric acid/10 mM $Na_2HPO_4$, adjusted pH to 4.4 with $H_3PO_4$.

Bovine Serum Albumin (BSA)-Citrate-Phosphate Buffer: BSA (Sigma) with low ß-hexosaminidase activity was dissolved in 100 ml of Citrate-Phosphate Buffer to give 0.6% BSA.

Substrates: 4-Methylumbelliferyl-ß-N-acetylglucosaminide (4-MUG) and 4-methylumbelliferyl-6-sulfo-ß-N-acetylglucosaminide (4-MUGS) from Toronto Research Chemicals Inc. (Toronto, ON) were prepared in Citrate-Phosphate Buffer to give a concentration of 3 mM.

Standard: 1 mM 4-Methylumbelliferone (4-MU, Toronto Research Chemicals Inc., Toronto) was prepared by dissolving 17.62 mg of 4-MU in 100 ml of Glycine-Carbonate Buffer. This was diluted 1000 times in Glycine-Carbonate Buffer to give 1 µM 4-MU. This stock was diluted further to prepare a standard curve of the fluorescent units versus five various concentrations of 4-MU in the range of 10-400 nM. A new standard curve was prepared for every assay.

Determination of Hex A & S Activity Using 4-MUGS as a Substrate. BSA (0.6%)-Citrate-Phosphate Buffer was added to an aliquot of cell extract (4-10 µg protein) to give a

final volume of 10 µl. This was mixed with 20 µl 3 mM 4-MUGS and incubated at 37°C for 30 min. The reaction was stopped by adding 970 µl of Glycine-Carbonate Buffer and mixing. The fluorescence generated by the enzymatic reaction was detected using a fluorescence spectrophotometer F-2000 (Hitachi) at an excitation of 364 nm and an emission of 448 nm. The blank was treated identically to the test samples except that 10 µl of BSA (0.6%)-Citrate-Phosphate Buffer was used instead of cell extract. Hex A & S activity was determined using the following formula:

$$\text{Hexosaminidase activity (nmol/hr/µg protein)} = \frac{\text{nmol of 4-MUGS hydrolysed}}{0.5\ (\text{hr}) \cdot \text{protein (µg)}}$$

ß-Hexosaminidase A Assay Using 4-MUG as a Substrate. Cell extract (16-40 µg protein) was mixed with BSA (0.6%)-Citrate-Phosphate Buffer to give a final volume of 40 µl. The mixture was then divided into two-20 µl aliquots. One aliquot was incubated at 52°C for 2 hr and the other was kept on ice as a non-heated control. After the heat treatment, a 10 µl aliquot was taken from the heat-treated and non-treated samples, and mixed with 20 µl of 3 mM 4-MUG. The reaction was allowed to proceed at 37°C for 30 min and stopped by adding 970 µl of Glycine-Carbonate Buffer. The activities of the heat-treated and non-treated samples were detected using the Hitachi F-2000 fluorescence spectrophotometer at an excitation of 364 nm and an emission of 448 nm. The blank was treated identically to the test samples without cell extract. The percent Hex A & S activity in the sample was calculated using the following formula:

$$\text{Hex A \& S \%} = \frac{\text{Fluorescence of (non-treated activity - heat-treated activity)} \cdot 100}{\text{Fluorescence of (non-treated activity)}}$$

2.17 ß-Galactosidase assay.

ß-Galactosidase activity was determined using 4-methylumbelliferyl-ß-galactoside (4-MU-ß-gal) as a substrate and following the method described by MacGregor *et al.* (1991).

Solutions:

Z-Buffer: 60 mM $Na_2PO_4$, 40 mM $NaH_2PO_4$, 10 mM KCl, 1 mM $Mg_2SO_4$, adjusted pH to 7.0 with NaOH.

4-MU-ß-gal Stock: 3 mM 4-MU-ß-gal was prepared by boiling in Z-Buffer until it dissolved (5-10 min). Aliquots (1 ml) of the stock solution were stored at -20°C until use. Before use, the stock solution was heat-treated again at 80°C until the substrate dissolved (~ 5 min).

Stop Buffer: 300 mM glycine, 15 mM EDTA, adjusted pH to 11.2 with NaOH.

To determine ß-galactosidase activity, a reaction mix containing an aliquot of cell extract (5-20 µg protein), Z-buffer to a final volume of 100 µl and 30 µl 4-MU-ß-gal stock solution was incubated at 37°C for 30 min. The reaction was stopped by mixing the reaction mix with 130 µl Stop Buffer. An aliquot of the mixture was used for detecting ß-galactosidase activity using the fluorescence spectrophotometer (F-2000) at an excitation of 364 nm and an emission of 448 nm. Glycine-Carbonate Buffer (0.17 M, pH 10.0) was used to dilute the reaction mixture, if necessary, and a reaction mix without cell extract was used as a blank. The specific ß-galactosidase activity (fluorescent units/30 min/µg of protein) were

used to estimate transfection efficiency. This was done by letting the average ß-galactosidase specific activity of the samples in a transfection equal one and converting the ß-galactosidase specific activity of each sample to a portion of one, this was used as a correction factor to normalize their corresponding co-expressed ß-hexosaminidase activity.

2.18 Separation of ß-hexosaminidase A, B and S isozymes.

A modified protocol, based on procedures by Mahuran *et al.* (1985) and Emiliani *et al.* (1990), was used to separate Hex A from Hex B and Hex S. A DEAE-cellulose (Pharmacia Biotech, Baie D'Urfe, PQ) column (1.4 ml bed volume) was equilibrated with 10 mM phosphate buffer, pH 6.0, and 400 to 900 µl (1.2 to 5.0 mg protein) of cell extract was loaded. After a 20 minute incubation at 4°C, the column was sequentially eluted with 6 ml of the phosphate buffer, 20 ml of a linear NaCl gradient (0-0.215 M), 5 ml of 0.215 M NaCl, 5 ml of 0.3 M NaCl and 5 ml of 0.5 M NaCl, all in the phosphate buffer. One ml fractions were collected and the activity in each fraction was measured using 4-MUG and/or 4-MUGS as the substrate. The fractions representing each isoenzyme form were pooled, dialysed, and concentrated; their identity was confirmed by Western blot analysis. The pooled and concentrated fractions corresponding to Hex A were used for $K_m/V_{max}$ determination.

2.19 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The method is based on that of Laemmli (1970) and the procedure described in "Molecular Cloning, A Laboratory Manual" by Sambrook *et al.* (1989).

Solutions:

10% Separating Gel: 10% acrylamide (acrylamide : bis-acrylamide = 29 : 1), 0.4 M Tris-HCl, pH 8.8, 0.1% SDS, 0.1% ammonium persulfate, 0.04% TEMED (N,N,N',N'-tetramethylethylenediamine).

5% Stacking Gel: 5% acrylamide (acrylamide : bis-acrylamide = 29 : 1), 0.125 M Tris-HCl, pH 6.8, 0.1% SDS, 0.1% ammonium persulfate, 0.04% TEMED.

4 x Sample Buffer: 200 mM Tris-HCl, pH 6.8, 400 mM Dithiothreitol (DTT), 8% SDS (electrophoresis grade), 0.4% bromophenol blue, 40% glycerol. This was stored at -20°C; an aliquot was thawed and mixed with 4 volumes of sample immediately before use.

4 x Running Buffer: 0.2 M Tris, 1.5 M glycine, 0.4% SDS without adjustment for pH.

Sample Preparation and Electrophoresis. A separating gel (0.075 x 10 x 6.5 cm) was cast using the 10% Separating Gel solution and allowed to polymerize for at least 1 hr. The 5% Stacking Gel solution was cast on top of the polymerized separating gel and polymerized for 1 hr. Cell extract (10-15 µl) was mixed with 3 µl 4 x Sample Buffer and placed in a boiling-water bath for 5 min. After a centrifugation at 10,000 x g for 5 seconds, the sample was cooled, loaded onto the polymerized 10% acrylamide separating gel/5% acrylamide stacking gel, and separated by electrophoresis in a Mini-PROTEAN II Electrophoresis Cell (Bio-Rad) at 200 volts (recommended by the manufacturer) for ~1 hr or until 5-10 min after the front dye ran off.

2.20 Discontinuous and high porous gradient SDS-PAGE.

Discontinuous high porous gradient SDS-PAGE, according to Doucet *et al.* (1988;

1990), was used to separate immunoprecipitated proteins derived from metabolic labelling experiments because it improved the separation of the precursor α-subunit from the precursor ß-subunit.

Solutions:

5% Acrylamide Gel Solution: 5% acrylamide (acrylamide:bisacrylamide=100:1), 0.4% SDS, 5% glycerol, 0.3 M glycine, 0.1 M Tris-HCl, pH 8.8, 0.15% ammonium persulfate, 0.05% TEMED.

12% Acrylamide Gel Solution: 12% acrylamide (acrylamide:bisacrylamide=100:1), 0.4% SDS, 5% glycerol, 0.3 M glycine, 0.1 M Tris-HCl, pH 8.8, 0.15% ammonium persulfate, 0.05% TEMED.

Stacking Gel Solution: 4% acrylamide (acrylamide:bisacrylamide=100:1), 0.4% SDS, 4 mM EDTA, 0.5% Glycerol and 0.07 M Tris-HCl, pH 6.8. The solution was degassed before the addition of 0.25% ammonium persulfate and 0.025% TEMED.

Running Buffer: 0.1% SDS, 100 mM Tris and 150 mM glycine without adjustment of pH.

Gel Preparation and Electrophoresis. A 5-12% gradient SDS-polyacrylamide separating gel (0.075 x 16 x 12 cm) was prepared in the casting stand of a PROTEAN II apparatus (Bio-Rad) by mixing 8.5 ml each of 5% and 12% Acrylamide Gel Solutions in a gradient gel maker. The gel was allowed to polymerize for a minimum of 1 hr and then the 4% stacking gel was cast. This also required at least 1 hr to polymerize. Protein samples (15-20 µl) were loaded onto the gel and separated by electrophoresis in a PROTEAN II apparatus (Bio-Rad). The electrophoresis was done at 300 volts (constant) in a cooling

system; the upper tank of the electrophoresis apparatus contained 1 x Running Buffer and the lower tank chamber contained 0.5 x Running Buffer.

2.21 Western blot.

Western blot analysis was used to assess the levels of α-subunit protein expressed in Cos-7 cells and/or fibroblasts.

Solutions:

Western Transfer Buffer (WTB): 0.19 M glycine, 20% methanol, 0.025 M Tris without adjustment of the pH.

Tris-Buffered Saline (TBS): 20 mM Tris, 0.5 M NaCl, adjusted pH to 7.5 with HCl.

TBST Buffer: 0.05% Tween 20 in the TBS buffer.

Antibodies:

The polyclonal antibody against human placental Hex A was a gift from Dr. Roy Gravel (McGill University, Montreal). The antibody was raised in rabbit by Dr. Greg Lee (University of British Columbia, Vancouver) using purified placental Hex A provided by Dr. Don Mahuran (University of Toronto).

The horseradish peroxidase-linked whole antibody raised in donkey against rabbit immunoglobulin (Amersham) was used as a secondary antibody with the Enhanced Chemi-Luminescence (ECL) system (Amersham).

Electrophoresis, Western Blot Transfer and Detection. Protein extracts from fibroblasts (20-40 μg) or from transfected Cos-7 cells (10-30 μg) were separated on a SDS-(10%)PAGE gel (0.075 x 6.5 x 10 cm, see section 2.19) in the Mini-PROTEAN II

Electrophoresis Cell at 200 volts until 5 min after the bromophenol blue dye had run off. The gel was removed from the Mini-PROTEAN II Electrophoresis Cell and the proteins in the gel were transferred to a nitrocellulose membrane. This was done by incubating the gel in 50 ml of WTB for 20 min with shaking then laying a WTB-pre-rinsed nitrocellulose membrane (5 x 8 cm, MSI) on top of the gel. Four pieces of WTB-pre-rinsed filter paper, cut to the same size as the membrane, were then laid on top of the membrane. The Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) was assembled according to the instructions from the manufacturer and the transfer was done in WTB with cooling, at 100 volts for 2 hr. The membrane was then removed and incubated at room temperature with shaking for 1 hr in 20 ml 5% skim milk in TBS buffer. After washing the membrane three times for 10 min in 20 ml TBST buffer, the membrane was ready for detection.

To detect the proteins of interest, the membrane was incubated with shaking in 15 ml of the anti-Hex A antibody (1:5000 dilution with TBST buffer) at room temperature for 1 hr and at +4°C overnight. This was followed by four washes in 20 ml TBST buffer. The membrane was then incubated in 15 ml of the secondary antibody, a 1:15,000 dilution of horseradish peroxidase-linked anti-rabbit immunoglobulin in TBST buffer, for 1 hr at room temperature with shaking. This was again followed by four washes in 20 ml TBST buffer. The protein-antibody complexes were detected using the ECL Western Blotting kit (Amersham) and following the protocol provided by the manufacturer. Briefly, after the final wash, the membrane was laid on a piece of clean plastic wrap and the residual solution was removed with a paper towel. This was immediately followed by incubating the membrane for 1 min in 2 ml of a mix of the reagent 1 and 2 (1 : 1) provided in the kit, and exposing the

membrane to REFLECTION™ autoradiography film (Du Pont) for 5-30 min.

2.22 Metabolic labelling.

Pulse-chase metabolic labelling was done to determine the effects of the benign mutations on the processing, targeting and stability of Hex A *in vivo*. A procedure based on that of Hasilik and Neufeld (1980a; 1980b) was developed.

Solutions:

Lysis Buffer: 10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P40, 0.02% $NaN_3$, 1 mM Pefabloc.

Wash Buffer A: 10 mM Tris-HCl, pH 8.6, 0.6 M NaCl, 0.1% SDS, 0.05% Nonidet P40.

Wash Buffer B: 10 mM Tris-HCl, pH 7.6, 150 mM NaCl.

Denaturing Solution: 20 mM Tris-HCl, pH 7.4, 1% SDS, 20 mM DTT.

Loading Buffer: 125 mM Tris-HCl, pH 6.8, 1% SDS, 10% Glycerol.

2.22.0 Labelling of cells.

Labelling of Fibroblasts. Prior to labelling with [$^{35}$S]methionine/[$^{35}$S]cysteine, a 20 x 100 mm tissue-culture dish of confluent fibroblasts was washed once with 4 ml of PBS, then incubated for 1 hr in 4 ml of methionine/cysteine-free minimum essential medium (met/cys-free medium, ICN Pharmaceuticals) supplemented with penicillin/streptomycin and 200 mM L-glutamine . For labelling, the cells were transferred to 2.9 ml of the met/cys-free medium supplemented with penicillin/streptomycin and L-glutamine, 150 µl of dialysed fetal bovine

serum (Sigma) and 30 µl (360 µCi) TRAN35S-LABEL (L-methionine [$^{35}$S] and L-cysteine [$^{35}$S], 1060-1090 Ci/mmol, ICN) and incubated for 3 hr.

Prior to labelling with $^{32}$P, fibroblasts were rinsed with 4 ml of phosphate-free medium (Dulbecco's Modified Eagle Medium, Gibco/BRL) supplemented with sodium pyruvate and L-glutamine and incubated for 1 hr in 3 ml of the same medium to deplete the intracellular phosphate. The cells were then labelled for 3 hr in 3.8 ml of the phosphate-free medium supplemented with sodium pyruvate and L-glutamine, 150 µl of dialysed fetal bovine serum, and 1 µl of $^{32}$P (500 mCi/ml, Du Pont).

In pulse-chase experiments, labelling was terminated by the addition of 4.0 ml of α-MEM supplemented with 10% fetal bovine serum, penicillin/streptomycin, 0.075 mg/ml L-methionine and 0.5 mg/ml L-cysteine. This was incubated for various intervals (ie. chase times) as indicated. In experiments that included protease inhibitors, 105 µM Leupeptin (Sigma) or 280 µM E-64 (L-trans-3-carboxyoxiran-2-carbonyl-L-leucylagmatine; Sigma) were present throughout the labelling and chase steps. In experiments with $NH_4Cl$ (Proia *et al.*, 1984), dialysed fetal bovine serum was not added in the labelling medium and 10 mM $NH_4Cl$ was present in the cell culture medium throughout the labelling and chase steps.

Labelling of Transfected Cos-7 Cells. Cos-7 cells were transfected with normal and mutant plasmid vectors as described in section 2.14. After transfection, the cells from 4 transfections with a single type of plasmid vector were pooled and aliquots of approximately $2 \times 10^6$ cells were distributed into 20 x 100 mm tissue-culture dishes. The cells were cultured in 20 ml of α-MEM supplemented with 10% fetal bovine serum and antibiotics and the media were changed at 24 hr. At 45 hr post-transfection, cells were treated as described for

labelling fibroblasts, except that 10 µl (150 µCi) TRAN35S-LABEL was used for the labelling.

2.22.1 Immunoprecipitation.

After labelling, cell and medium extracts were prepared as described in section 2.15.1. Prior to immunoprecipitation, fibronectin was removed from the cell/medium extracts to minimize the non-specific radioactive background in the immunoprecipitated materials. For fibroblast cell extracts, 0.5 ml of cell extract containing an equal amount of protein (1.2 or 1.4 mg) in Lysis Buffer was incubated with 4 µl rabbit anti-human fibronectin antiserum (Gibco/BRL) at 4°C for 1 hr with agitation. The antibody-fibronectin complexes were removed from the cell extract with 70 µl of PANSORBIN™ cells (CALBIOCHEM, La Jola, CA), which had been prewashed with Lysis Buffer plus 1% BSA. After an incubation for 15 min at 4°C with agitation, the sample was centrifuged at 12,000 x g for 2 min. The PANSORBIN precipitation was repeated three times and the resulting supernatant was used for immunoprecipitation. For medium extracts, the procedure was the same as described above. For Cos-7 cell extracts, the procedure was the same as that for fibroblast extracts, except that the cell extracts were directly treated with the pre-washed PANSORBIN without the addition of anti-fibronectin antibody.

Immunoprecipitation of Hexosaminidase Using Three Antibodies. Three forms of the α-subunit of ß-hexosaminidase, (ie. free, ß-subunit-associated, and total), were immunoprecipitated using the following antibodies, anti-Hex A, anti-α-subunit and anti-Hex B. The anti-α-subunit and anti-Hex B specific polyclonal antibodies were prepared in goat

and a gift from Dr. Rick Proia, NIH (Hasilik and Neufeld, 1980a). After removing the fibronectin, the supernatant (1 ml) was divided into three portions. Each portion (0.33 ml) was mixed with 2 µl of antibody (anti-Hex A, anti-α-subunit or anti-Hex B) and incubated at 4°C overnight with rocking. The solution containing the immune complexes was then incubated with 70 µl of prewashed PANSORBIN cells at 4°C for 1 hr with rocking and centrifuged at 12,000 x g for 2 min. The immune complex-PANSORBIN pellet derived from the cell extracts was washed once with 900 µl of Wash Buffer A and once with 1 ml of Wash Buffer B. Pellets derived from the medium extracts were washed twice with Wash Buffer A and once with Wash Buffer B. After washing, the pellet was resuspended in 50 µl of Loading Buffer, incubated at 100°C for 5 min to release the immunocomplexes from PANSORBIN cells, and centrifuged at 12,000 x g for 2 min. The supernatant was mixed with 10 µl of 0.1 M DTT and 1 µl 0.15% bromophenol blue and incubated again at 100°C for 5 min. After a 5 second centrifugation, 20 µl of the sample was loaded on a high porous 5-12% gradient SDS gel (0.075 x 12 x 16 cm) and separated by electrophoresis at 300 volts as described in the section 2.20.

After electrophoresis, the signals of the $^{35}$S-labelled proteins of interest were visualized by fluorography. Briefly, the gel was incubated twice for 30 min in 50 ml fixing solution (acetic acid : methanol : $H_2O$ = 10 : 20 : 70). The radioactive signals were enhanced by incubating the gel for 40 min in 30 ml of DUPONT ENTENSIFY solution A and then B (NEN Biotechnology System, Boston, MA). The gel was then dried under vacuum and was exposed to a Kodak X-OMAT-AR film for 1-10 days.

Autoradiography was used to detect the signals of the $^{32}$P-labelled proteins. Once

again, the gel was incubated for 30 min in 50 ml of the fixing solution; this was repeated twice. The gel was then dried under vacuum and was exposed to a Kodak X-OMAT-AR film at -80°C, with an intensifying screen, for one week.

Immunoprecipitation of the α-subunit of ß-Hexosaminidase Using Anti-α-subunit Antibody. To obtain immunoprecipitated α-subunit without interference from the ß-subunit, a two-step protocol was developed to immunoprecipitate only the α-subunit in both its precursor and mature forms.

(1) Primary Immunoprecipitation. To immunoprecipitate both the free and associated forms of the α-subunit, anti-Hex A antibody (2 µl) was mixed with equal amounts of fibroblast extract (1.2 mg protein) that were adjusted to the same volume (600 µl) with Lysis Buffer. The mix was incubated overnight at 4° C with rocking. The antibody-antigen complexes were precipitated by an incubation for 1 hr with 70 µl pre-washed PANSORBIN followed by centrifugation at 12,000 x g for 2 min. The whole process was repeated once to completely recover the α-subunit. To release the antibody-antigen complexes from the PANSORBIN cells, and to dissociate the α-subunit from the ß-subunit, the pellets derived from the two centrifugations were resuspended in 50 µl of Denaturing Solution and incubated at 100°C for 10 min. This was centrifuged at 12,000 x g for 4 min, the supernatant was transferred to a fresh tube and the denaturation step was repeated once. The supernatants obtained from the two centrifugations were combined and mixed with 0.9 ml Lysis Buffer containing 1% BSA and 4 mM N-ethylmaleimide (NEM, Sigma, Mississauga, ON) to dilute the SDS concentration in the Denaturing Solution. NEM was used as a chelator of DTT in the Denaturing Solution.

(2) Secondary Immunoprecipitation. The free and dissociated α-subunit in the supernatant (1 ml) were immunoprecipitated by incubation with 4 µl anti-α-subunit antibody overnight at 4°C with rocking. This was followed by an incubation with 70 µl pre-washed PANSORBIN for 1 hr at 4°C with rocking and centrifugation at 12,000 x g for 5 min. The pellet was kept on ice and the supernatant was treated again with 70 µl prewashed PANSORBIN followed by centrifugation at 12,000 x g for 5 min to completely recover the immune complexes. The two pellets obtained from two centrifugations were suspended, combined, washed once with 1 ml Wash Buffer A and once with 1 ml Wash Buffer B. The pellets were then resuspended in 50 µl of Loading Buffer, incubated at 100°C for 5 min, and centrifuged at 12,000 x g for 2 min. The supernatant containing the immunocomplexes released from the PANSORBIN cells was mixed with 10 µl of 0.1 M DTT and 1 µl 0.15% bromophenol blue, and incubated again at 100°C for 5 min. After a centrifugation for 5 seconds, 20 µl of the sample was loaded on a high porous 5-12% gradient SDS gel (0.075 x 12 x 16 cm) and separated by electrophoresis at 300 volts as described in the section 2.20. The labelled proteins were detected by fluorography as described above.

# 3. RESULTS

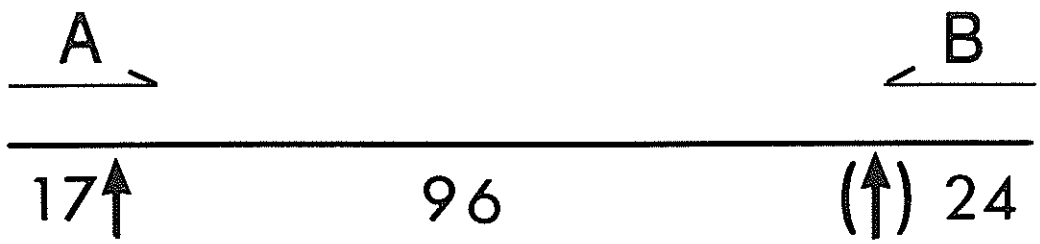
The primary aims of these studies were to characterize benign mutation-containing Hex A and to determine the effects of the two benign mutations, C739T(R247W) and C745T(R249W), on Hex A activity. When the experiments described within this section were initiated, a second apparently benign mutation had just been identified in the laboratory. SSCP analysis of the genomic DNA of the parent of a Hex A pseudodeficient proband had revealed a shift in exon 7 of the *HEXA* gene. Direct sequencing showed that a C745T mutation, that would result in an R249W substitution in the protein product, was present. Our initial goals were to confirm the presence of this specific change in the pseudodeficient proband and her family and to determine the frequency of this mutation in carrier populations. Our efforts were then focused on determining if the two benign mutations, C739T(R247W) and C745T(R249W), were responsible for Hex A deficiency, and if so, how they led to this deficiency.

3.0 Confirmation and frequency estimation of the C745T(R249W) mutation.

To (1) confirm the benign mutation C745T(R249W) to be an inherited mutation, and not a new mutation or artifact introduced during the identification process, and (2) to estimate the frequency of this mutation in the Ashkenazi Jewish and non-Jewish populations, a strategy to specifically detect this mutation was designed (Fig.2a). Although the C745T change destroys a *Hpa*II site, this did not provide a specific diagnostic test because many mutations could result in the loss of the same restriction enzyme site. To allow specific detection of the C745T mutation, two oligonucleotide primers, each carrying a single base change, were used to PCR-amplify the C745T-containing region of exon 7. The base change in primer B

Fig.2. Strategy for the detection of the C745T mutation. a. Arrows indicate *Dde*I sites and the parentheses indicate the site created in the presence of the C745T mutation. The asterisks under primers A and B indicate the single base changes introduced to create a control *Dde*I site in primer A and to create a *Dde*I site in the presence of the C745T change when primer B is used in the PCR reaction. b. Agarose gel electrophoresis of the products of the strategy used with the A and B primers. Lane 1, Normal DNA cut by *Dde*I; Lane 2, The proband (16819) who had a C745T mutation on one allele and a G805A mutation on the other allele; Lane 3, The proband's mother who carried a G805A mutation; Lane 4, The proband's father who carried the C745T mutation; Lane 5, Normal DNA uncut.

a.




Total size-137 bp

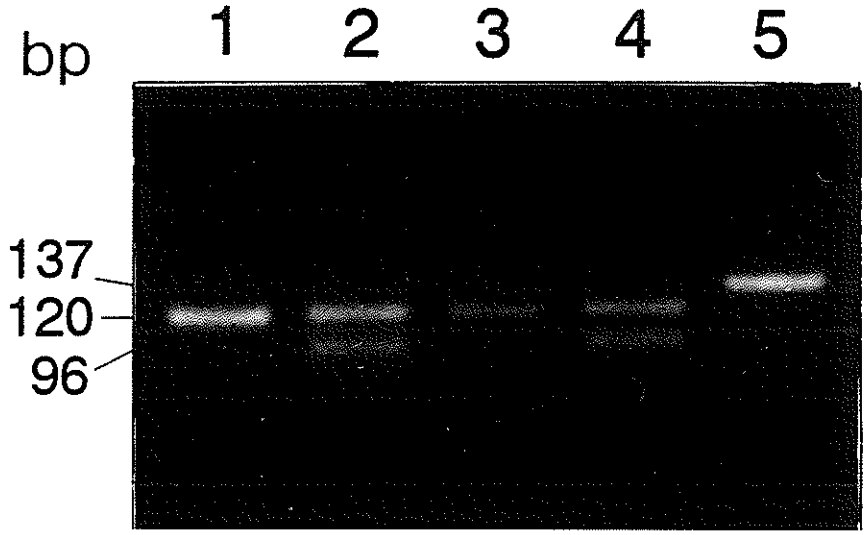
A-5' TTCAGGAAGTGTGAACCTGAGGG 3'

*

B-5' CTCTGCAAGCACACGGATACCTC 3'

*

b.



(Fig.2a) created a *Dde*I site in the PCR product only in the presence of the C745T mutation. A base change in primer A (Fig.2a) created a *Dde*I site that was present in all PCR products, acting as a positive control to determine if the restriction enzyme had cut the product.

Using the strategy described above (Fig.2a), the presence of the C745T mutation was tested for in genomic DNA samples from the proband (16819) and her parents. The presence of a 96 bp band indicated that a *Dde*I site had been created by the C745T mutation in the PCR product of the proband and her father (Fig.2b). The 96 bp band was not present in the PCR product from the mother, or the normal control. These results confirmed the presence, and the inherited nature, of the C745T mutation in this proband. This suggested that the C745T(R249W) mutation on one allele, together with the G805A(G269S) disease-causing mutation previously detected on the other allele, were responsible for pseudodeficiency in this proband.

Ashkenazi Jewish and non-Jewish enzyme-defined carriers, obligate carriers, and non-carriers of Tay-Sachs disease, identified using the synthetic substrate 4-MUG (Kaback *et al.*, 1977a and 1977b), were tested for the presence of the C745T mutation using the strategy described above. A summary of the results is shown in Table 1. The C745T mutation was found in 4 of 63 non-Jewish enzyme-defined carriers. Using PCR-based detection strategies (Triggs-Raine *et al.*, 1991; Triggs-Raine *et al.*, 1995), these DNA samples were also tested for other benign and disease-causing mutations. These included the C739T benign mutation (Triggs-Raine *et al.*, 1992), the G→A change at intron 9+1, common in non-Jewish populations (Akli *et al.*, 1991; Akerman *et al.*, 1992; Landels *et al.*, 1992), and three disease-causing mutations common in the Ashkenazi Jewish population (Paw *et al.*, 1990; Triggs-

Table 1. DNA Analysis of Tay-Sachs Disease Carriers and Noncarriers.

| Heritage and Status | Total Number Tested | Number (%) of C745T |
|---|---|---|
| Non-Jewish: | | |
| Carriers[a]---------- | 63[b] | 4 (6%) |
| Noncarriers[a]---------- | 10 | 0 |
| Obligate Carriers---- | 6 | 0 |
| Ashkenazi Jewish: | | |
| Carriers[a]---------- | 218[c] | 0 |
| Noncarriers[a]---------- | 10 | 0 |

[a] Status of sample is based on an enzyme assay with the synthetic substrate, 4-MUG.

[b] Of these 63, 19 were examined in a previous study (Tomczak et al., 1993); 14 of these 19 were shown to have a previously described mutation and thus were not tested for C745T; the remaining 44 samples were tested only for the C745T mutation, unless they were known to be C745T positive.

[c] Of these 218, 209 were examined in a previous study (Tomczak et al., 1993); 197 of these 209 were shown to have a previously described mutation and thus were not tested for C745T; the remaining 9 samples were tested only for the C745T mutation.

Raine *et al.*, 1990; Grebner and Tomczak 1991; Landels *et al.*, 1991). The three disease-causing mutations common among Ashkenazi Jews included the G→C substitution at the intron 12 + 1 junction (Arpaia *et al.*, 1988; Myerowitz, 1988), a 4-bp insertion at position 1278 of exon 11 (Myerowitz and Costigan, 1988), and a G→A substitution at position 805 of exon 7 (Navon and Proia, 1989; Paw *et al.*, 1989). These mutations were not detected in the C745T mutation-containing DNA samples. The C745T mutation was not detected in any of the other sample groups, including the enzyme-defined Jewish carriers. The previously reported C739T benign mutation and the C745T benign mutation accounted for about 36-38% of enzyme-defined carriers in the non-Jewish population (Triggs-Raine *et al.*, 1992; Cao *et al.*, 1993; Kaback *et al.*, 1993)

3.1 Expression of the benign mutations, R247W and R249W, in Cos-7 cells.

To determine if the benign mutations, C739T(R247W) and C745T(R249W), could cause Hex A deficiency, and were not simply on the same allele as another mutation and therefore associated with Hex A deficiency, further studies were required. We decided to introduce the benign mutations into the α-subunit cDNA and to express the mutant protein in Cos-7 cells. This would allow us to determine if the mutations could cause Hex A deficiency. This same approach could then be used to produce the enzyme for study and to provide a system in which to study the processing of the α-subunit containing the mutations that result in Hex A deficiency.

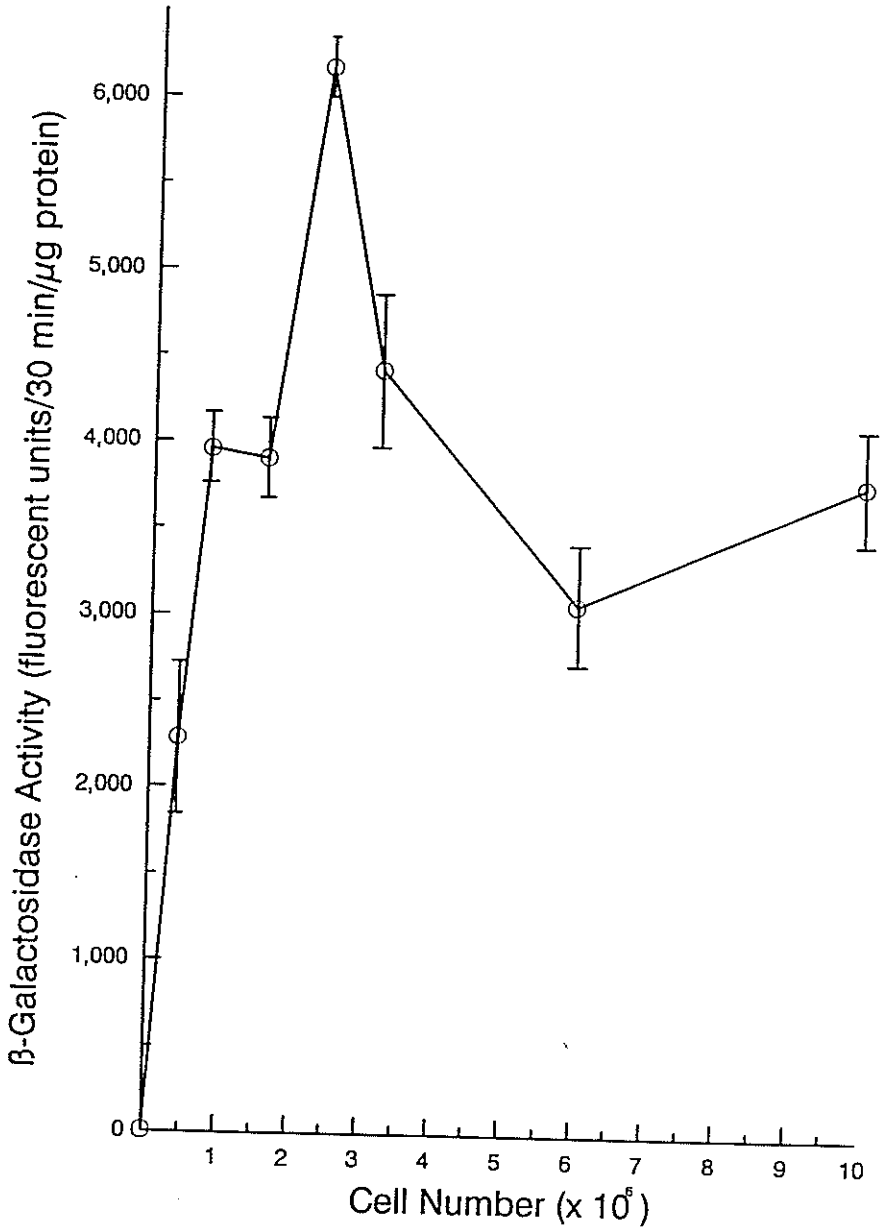
3.1.0 Optimization of transfection conditions for Hex S and Hex A expression.

Our first step was to identify appropriate conditions for the transient expression of the α-subunit to form Hex S (αα) and Hex A (αß) in Cos-7 cells. DNA was transfected into Cos-7 cells by electroporation using several different conditions to look for those under which ß-hexosaminidase expression was well above the endogenous activity of ß-hexosaminidase in Cos-7 cells.

Initially, we determined the appropriate concentration of Cos-7 cells to use in the transfection. Various concentrations of Cos-7 cells were transfected with 2 μg of the ß-galactosidase expression vector, pRc/CMV-ß-gal. We chose to use 2 μg of the pRc/CMV-ß-gal vector because preliminary experiments had shown 2 μg of DNA expressed sufficient ß-galactosidase activity for detection without affecting the expression of protein from co-transfected vectors. The levels of expressed ß-galactosidase activity using the various concentrations of Cos-7 cells are shown in Fig.3. The highest level of expressed ß-galactosidase activity was observed when 2.5 x $10^6$ to 3.0 x $10^6$ cells in 400 μl were used in the electroporation. The data shown in Fig. 3. were derived from one experiment; a similar optimal cell number was previously reported by Chang *et al.* (1991) and observed in this laboratory (T. Salo, personal communication). The cell concentration of 3.0 x $10^6$ cells/400 μl was chosen for use in subsequent transfection experiments.

The next experiments were done to determine the concentration of the α-subunit expression vector, αpSVL, that expressed a level of Hex S (αα) well above the endogenous level of ß-hexosaminidase in Cos-7 cells. Various concentrations of αpSVL were transfected into Cos-7 cells and the expressed Hex S activities were measured using 4-MUG as the substrate. The basal activity of Cos-7 cells towards 4-MUG, 420 nmol/hr/mg protein, was

Fig.3. Estimation of optimal cell number for transfection by electroporation. Various numbers of Cos-7 cells, as shown, were suspended in 400 µl PBS and electroporated with 2 µg of the ß-galactosidase expression vector, pRc/CMV-ß-gal. The culture medium was changed at 24 hr post-transfection and the cells were harvested at 72 hr. Cell extracts were prepared and used for the determination of protein concentration and ß-galactosidase activity. The expressed ß-galactosidase activities, after subtracting Cos-7 endogenous activity (4 fluorescent units/30 min/µg protein), are shown. The data were derived from a single experiment with duplicate samples at each point. For each sample the assays for protein concentration and ß-galactosidase activity were done in duplicate. Each point represents the average specific activity of the two samples and the bar represents the activity range of the two samples.


β-Galactosidase Activity (fluorescent units/30 min/μg protein)
6,000
5,000
4,000
3,000
2,000
1,000
0
1
2
3
4
5
6
7
8
9
10
Cell Number (x 10⁵)

subtracted from the expressed Hex S activity. The highest level of expressed Hex S activity (~3,000 nmol/hr/mg protein) was reached using between 5.0 and 7.0 μg of αpSVL DNA in the transfection (Fig.4). For subsequent experiments, 6.5 μg of αpSVL was used in the transfections and was confirmed to be optimal for ß-hexosaminidase expression.

Because Hex A is comprised of one α- and one ß-subunit, co-transfection of the α- and ß-subunit expression vectors was required to generate Hex A. We wanted to find an appropriate concentration and ratio of αpSVL to the expression vector carrying the ß-subunit of ß-hexosaminidase, pCD43, for the expression of Hex A in Cos-7 cells. Because both α-subunit homodimers (Hex S) and αß-subunit heterodimers (Hex A) would be formed, we looked for conditions where a substantial amount of Hex A would also be formed. Given that Hex A has a higher $V_{max}$ than Hex S toward 4-MUG, this was predicted to be where the total Hex A & S activity and the % Hex A and S were at their highest. The concentration of the ß-subunit expression vector, pCD43, that would yield the highest level of expressed ß-hexosaminidase activity when transfected with 5 μg αpSVL into Cos-7 cells, was determined. Two separate experiments were done and the expressed ß-hexosaminidase activity was measured using 4-MUG as the substrate. The endogenous levels of Cos-7 ß-hexosaminidase are shown on the Y-axis at the zero point of pCD43. The highest Hex A & S activity and percent Hex A & S of the total ß-hexosaminidase activity were reached when 2.3 to 3.5 μg of pCD43 DNA were used for transfection (Fig.5). Therefore using 5 μg of αpSVL, and approximately 3 μg of pCD43 appeared to yield the highest level of Hex A activity.

To determine an appropriate αpSVL concentration for the expression of Hex A, experiments similar to those described above were done by co-transfecting different amounts

Fig.4. Determination of αpSVL concentration for Hex S (αα) expression in Cos-7 cells. Various amounts of αpSVL were mixed with 3.0 x $10^6$ Cos-7 cells in 400 µl PBS. After electroporation, the culture medium was changed at 24 hr and the cells were harvested at 72 hr. Cell extracts were prepared and used for the measurement of protein concentration and ß-hexosaminidase activity using 4-MUG as the substrate. The expressed specific Hex S activities at different αpSVL concentrations are shown after subtracting the endogenous activity in Cos-7 cells. The data represent two separate experiments with duplicate samples at each point. Assays for protein concentration and ß-hexosaminidase activity were done in duplicate for each sample. Each point represents the average specific activity of the samples and the bar represents sample standard deviation of the specific activity in these samples.

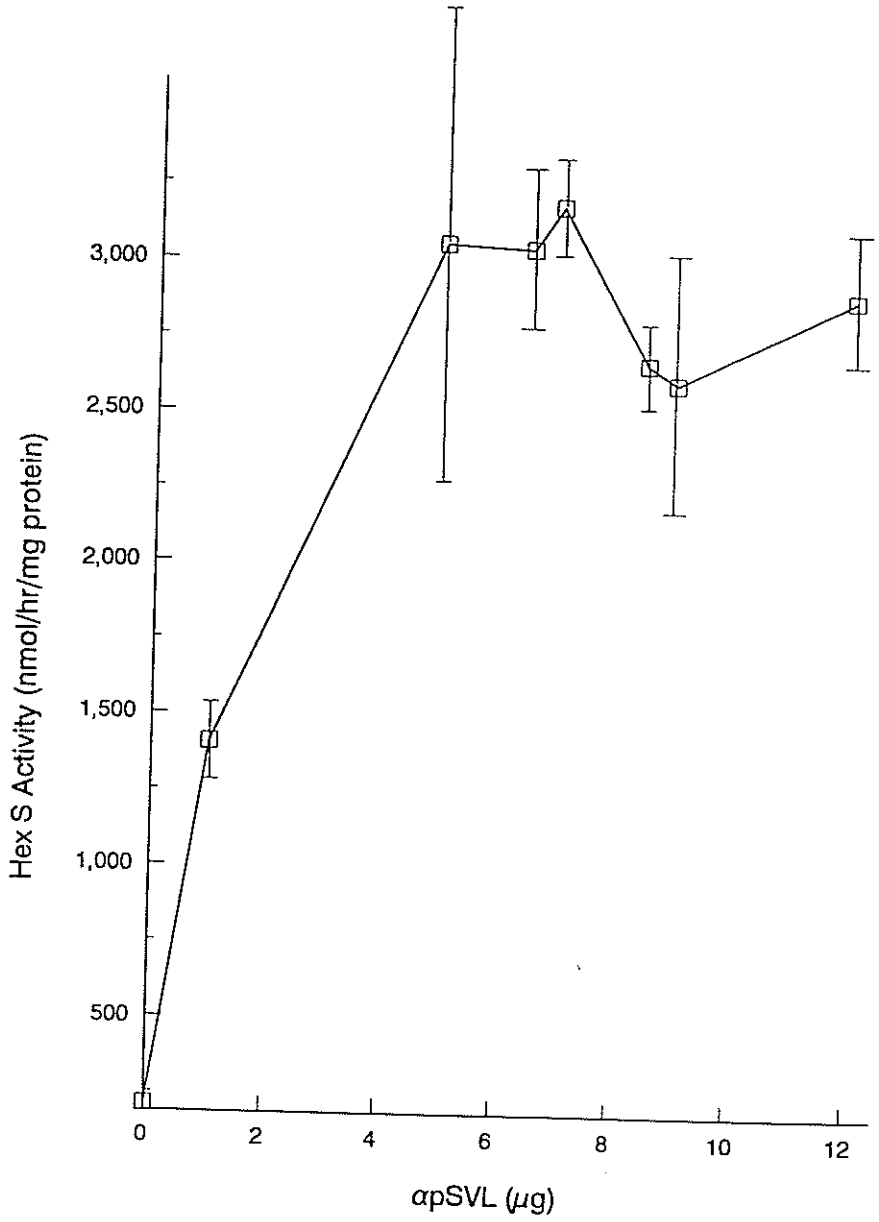
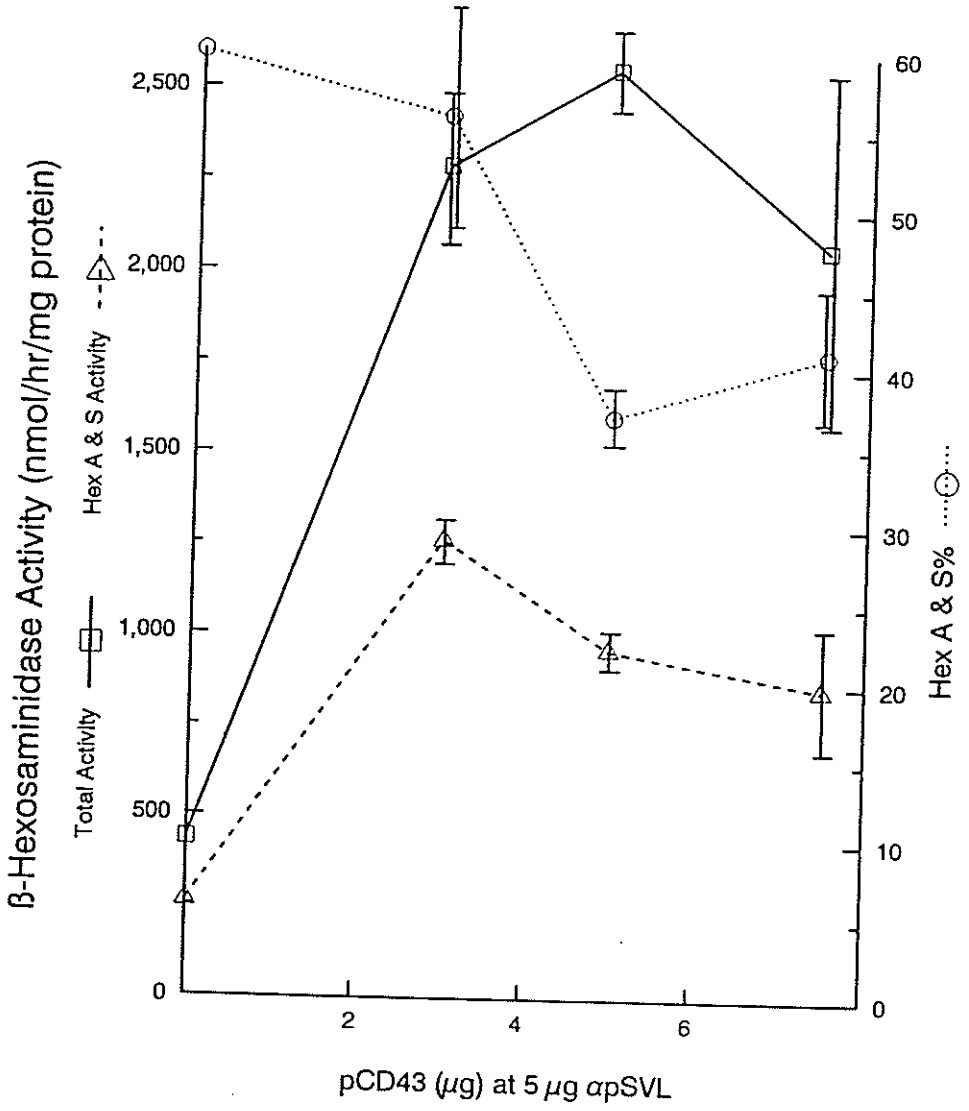

Hex S Activity (nmol/hr/mg protein)
3,000
2,500
2,000
1,500
1,000
500
0
2
4
6
8
10
12
αpSVL (μg)

Fig.5. Estimation of pCD43 concentration for Hex A (αß) expression in Cos-7 cells. Various amounts of pCD43 were electroporated with 5 µg αpSVL into 3.0 x $10^6$ Cos-7 cells in 400 µl PBS. No αpSVL was transfected at the zero point. The medium was changed at 24 hr post-transfection and the cells were harvested at 72 hr. The cell extracts were prepared and used for the determination of the protein concentration and ß-hexosaminidase activity using 4-MUG as the substrate. The total ß-hexosaminidase activity was determined before and after a heat treatment. The Hex A & S activities were calculated by subtracting the activity that remained after a treatment of the cell extract at 52°C for 2 hr, Hex B, from the total activity (see section 2.16). The data represent two experiments with duplicate samples at each point and duplicate assays for protein concentration and activity for each sample. The endogenous ß-hexosaminidase activity of Cos-7 cells is shown at the zero point of pCD43. Each point represents the average specific activity of the duplicate samples and the bar represents the sample standard deviation of the specific activity in these samples. The total and Hex A & S specific activities are indicated on the left Y axis. The apparent percentage of the Hex A & S activity is indicated on the right Y axis.


ß-Hexosaminidase Activity (nmol/hr/mg protein)
Total Activity
Hex A & S Activity
Hex A & S%
2,500
2,000
1,500
1,000
500
0
60
50
40
30
20
10
0
2
4
6
pCD43 (μg) at 5 μg αpSVL

of αpSVL with a constant amount, 3.0 μg, of pCD43. The expressed ß-hexosaminidase activities, from two separate experiments, were determined using 4-MUG as the substrate. The results are shown in Fig.6. The highest Hex A & S activity was obtained when 6.0 to 7.0 μg of αpSVL was co-transfected with 3 μg of pCD43. At this point, the percent Hex A & S had reached a plateau and appeared to be dropping.

Another experiment was done to examine the effect of the αpSVL to pCD43 ratio on Hex A expression. As shown in Fig.7, the ratio of αpSVL to pCD43 and the total DNA concentration in the transfections varied. The expressed ß-hexosaminidase activities were determined using 4-MUG as the substrate. Hex A & S activity and percent Hex A & S were at their highest level when the ratio was between 2.2 : 1 (9.5 μg, ie. αpSVL = 6.5 μg and pCD43 = 3.0 μg) and 2.8 : 1 (8.8 μg, ie. αpSVL = 6.5 μg and pCD43 = 2.3 μg) of αpSVL to pCD43. The amount of αpSVL that resulted in the highest Hex S and Hex A expression was 6.5 μg, as had been shown in the previous experiments (Fig.4 and Fig.6). The amount of pCD43, 2.3-3.0 μg, that appeared to yield the best Hex A expression in the previous experiments (Fig.5), also appeared to give the highest Hex A expression in this experiment (Fig.7).

We chose then to determine the effect of the total DNA concentration of both αpSVL and pCD43 on Hex A expression by co-transfecting various amounts of αpSVL and pCD43 at the ratio of 2.8 : 1. The results of two separate experiments are shown in Fig.8. The total ß-hexosaminidase activity, Hex A & S activities and percent Hex A & S, continuously increased with increasing DNA concentration until a maximum of 7.0 μg was reached. The activity appeared to reach a plateau at this point, and the activity was only slightly increased

Fig.6. Estimation of αpSVL concentration for expression of Hex A & S in Cos-7 cells. Various amounts of αpSVL (0 to 7.5 μg) and 3 μg pCD43 were electroporated into 3.0 x $10^6$ Cos-7 cells in 400 μl PBS. The medium was changed at 24 hr post-transfection and the cells were harvested at 72 hr. The cell extracts were prepared and used to determine the protein concentrations and ß-hexosaminidase activities using 4-MUG as a substrate. The Hex A & S activities were determined by subtracting the activity which remained after a treatment of the cell extract at 52°C for 2 hr, Hex B, from the total activity (see section 2.16). The data are derived from two separate experiments with duplicate samples at each point and duplicate assays for protein concentration and ß-hexosaminidase activity for each sample. Each point represents the average specific activities of the samples in the experiments and the bar represents the sample standard deviation of the specific activity in these samples. The apparent percentage of the Hex A & S activity is indicated on the right Y axis.

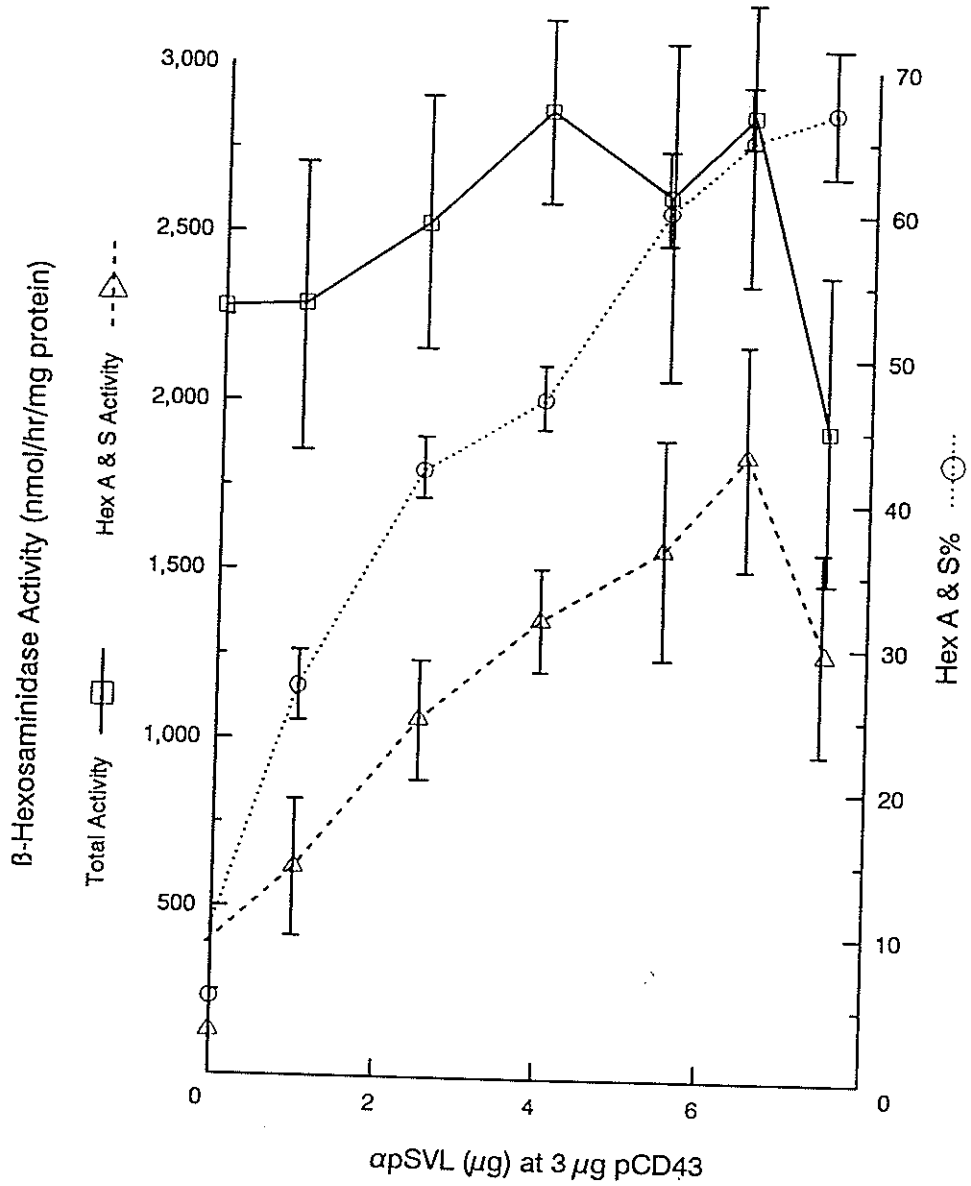

ß-Hexosaminidase Activity (nmol/hr/mg protein)
Total Activity
Hex A & S Activity
Hex A & S%
3,000
2,500
2,000
1,500
1,000
500
0
70
60
50
40
30
20
10
0
2
4
6
αpSVL (μg) at 3 μg pCD43

Fig.7. Effect of the ratio of αpSVL to pCD43 on the expression of Hex A in Cos-7 cells. Various amounts of αpSVL and pCD43 were electroporated into 3.0 x $10^6$ Cos-7 cells in 400 µl PBS. The medium was changed at 24 hr post-transfection and cells were harvested at 72 hr. Cell extracts were prepared and used for the determination of protein concentration and ß-hexosaminidase activity toward 4-MUG. The Hex A & S activities were determined by subtracting the activity which remained after a treatment of the cell extract at 52°C for 2 hr, Hex B, from the total activity (see section 2.16). Two separate experiments were performed with duplicate samples at each point and duplicate assays for protein concentration and ß-hexosaminidase activity for each sample. The endogenous ß-hexosaminidase activity of Cos-7 cells is shown at the zero point. Each point represents the average specific activity of the samples of the experiments and the bar represents the sample standard deviation of the specific activity in these samples. The apparent percentage of the Hex A & S activity is indicated on the right Y axis.

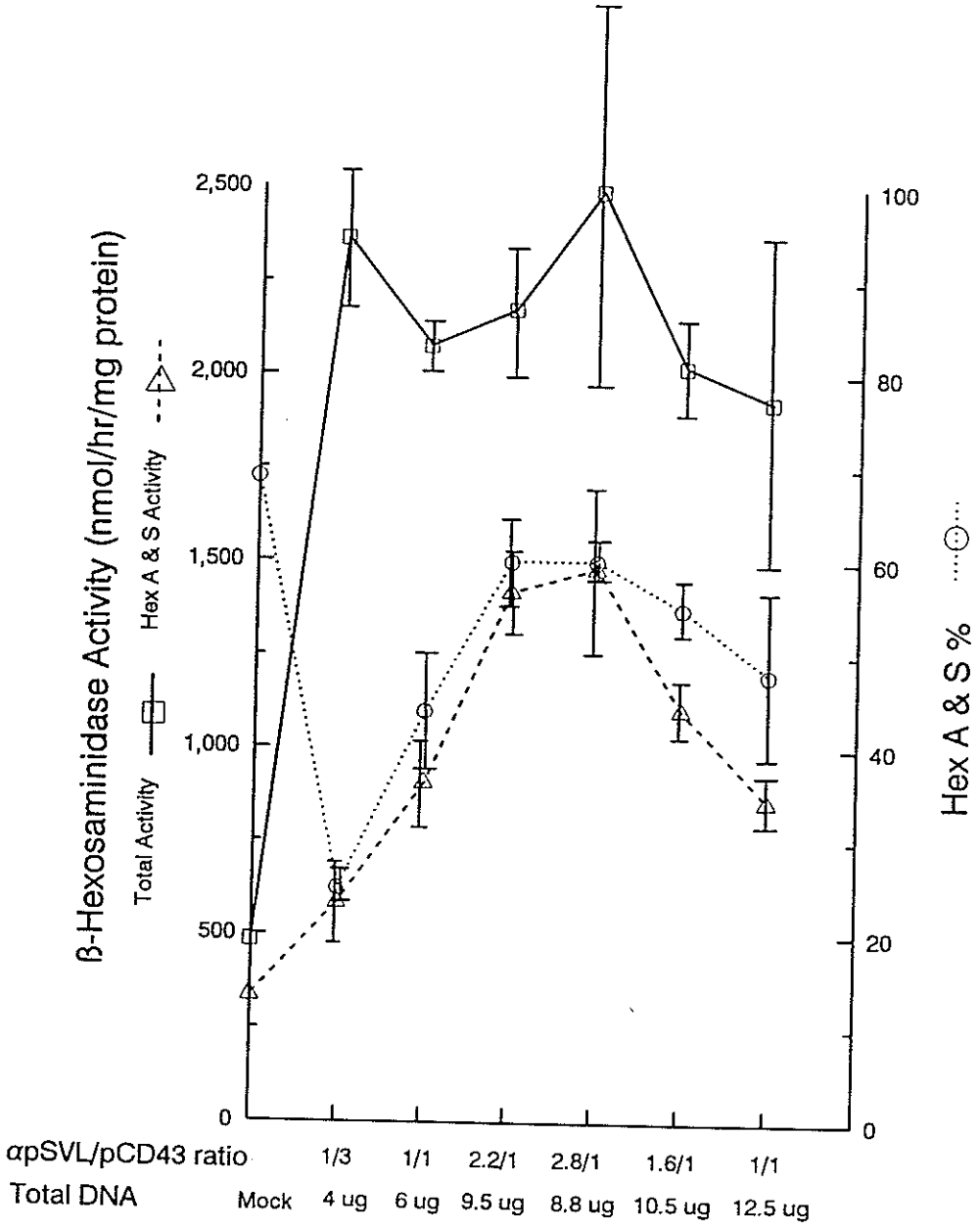
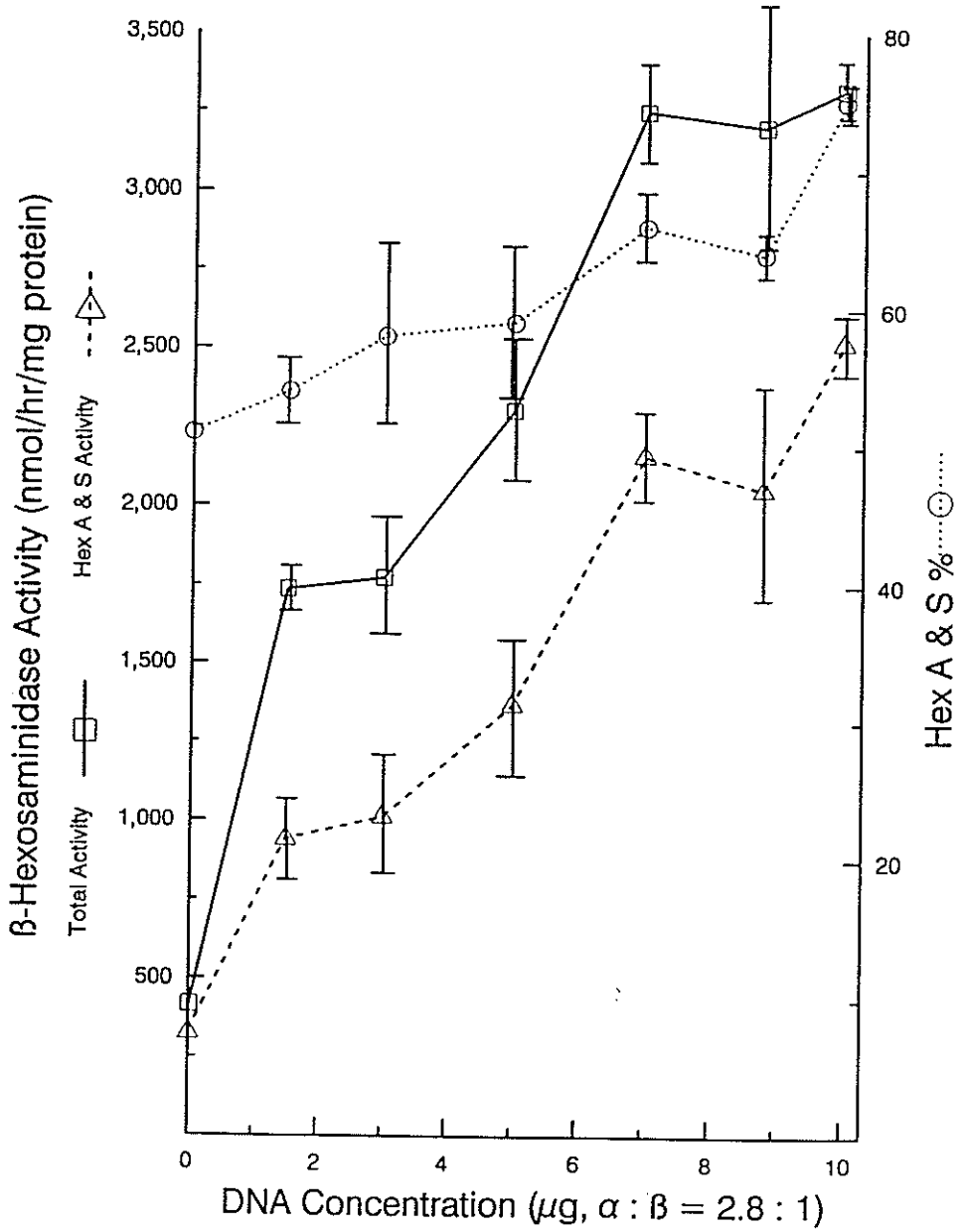

β-Hexosaminidase Activity (nmol/hr/mg protein)
Total Activity
Hex A & S Activity
Hex A & S %
2,500
2,000
1,500
1,000
500
0
100
80
60
40
20
0
αpSVL/pCD43 ratio
1/3
1/1
2.2/1
2.8/1
1.6/1
1/1
Total DNA
Mock
4 ug
6 ug
9.5 ug
8.8 ug
10.5 ug
12.5 ug

Fig.8. Examination of the effect of DNA concentration on Hex A & S expression in Cos-7 cells. Various amounts of αpSVL and pCD43, 1.5 μg, 3.0 μg, 5.0 μg, 7.0 μg, 8.8 μg, and 10.0 μg, at a constant ratio of αpSVL : pCD43 = 2.8 : 1, were electroporated into $3.0 \times 10^6$ Cos-7 cells in 400 μl PBS. Cell extracts were prepared and used for the determination of protein concentration and ß-hexosaminidase activity levels. Total ß-hexosaminidase, Hex A & S activities and percentage of Hex A & S were determined (see section 2.16). Two experiments were performed with duplicate samples at each point and duplicate assays for protein concentration and ß-hexosaminidase activity for each sample. The endogenous ß-hexosaminidase activity of Cos-7 cells is shown at the zero point DNA concentration. Each point represents the average specific activity of the samples of the experiment and the bar represents the sample standard deviation of the specific activity in these samples. The apparent percentage of the Hex A & S activity is indicated on the right Y axis.


ß-Hexosaminidase Activity (nmol/hr/mg protein)
Total Activity
Hex A & S Activity
Hex A & S %
3,500
3,000
2,500
2,000
1,500
1,000
500
80
60
40
20
0
2
4
6
8
10
DNA Concentration (μg, α : ß = 2.8 : 1)

when a total DNA concentration of 10 μg was used.

In summary, based on the data obtained from the above series of experiments, the conditions chosen for Hex S and Hex A & S expression in Cos-7 cells by electroporation were: 3.0 x $10^6$ Cos-7 cells/400 μl per transfection, 6.5 μg αpSVL for Hex S expression, and 6.5 μg αpSVL and 2.3 μg pCD43 for Hex A & S expression. While these conditions did not yield a complete optimization for the transfection conditions, they provided a strategy that produced levels of Hex S and Hex A & S expression considerably higher than the basal levels in Cos-7 cells.

3.1.1 Preparation of vectors for the expression of benign and disease-causing mutations.

To study the effects of benign mutations on Hex S and Hex A using the transient expression system in Cos-7 cells, mutant expression vectors containing the benign mutations were also required. In addition, vectors expressing varying levels of ß-hexosaminidase associated with infantile, juvenile, and adult-onset forms of $G_{M2}$ gangliosidosis were required as controls.

To express ß-hexosaminidase harbouring the α-subunit carrying the benign mutations, R247W and R249W, in Cos-7 cells and to determine the effects of these mutations on this enzyme, the C739T(R247W) and C745T(R249W) changes were introduced into the α-subunit cDNA and subcloned into pSVL. To provide α-subunit cDNA harbouring disease-causing mutations as controls, αpSVL carrying G805A(G269S)-, G749A(R250D)- or C508T(R170W)-containing cDNAs, associated with adult-onset, juvenile and infantile forms of $G_{M2}$ gangliosidosis, respectively, were also prepared. The severity of the clinical phenotype

associated with mutations has been shown to be inversely correlated with the level of residual Hex A activity produced by a mutant allele (Conzelmann *et al.*, 1983).

To generate the various mutant αpSVL derivatives three methods of site-directed mutagenesis were used. To prepare the mutant vector, C739TαpSVL, a combination of PCR and subcloning techniques were used (see section 2.12.0). The presence of the C739T change, but no others, was confirmed by others (Triggs-Raine, unpublished).

To produce the C745TαpSVL mutant vector, a modified USE method was used (see section 2.12.1). The mutant strain *BMH*71-18 was used as a host for the transformation of the reaction mix to amplify the mutant plasmid DNA and to avoid correction of the introduced mismatched base. To directly screen bacterial colonies for the presence of C745TαpSVL, a different PCR-based strategy from the previously-described strategy (see section 3.0 and Fig.2) was used. Briefly, two primers, NEB29 and NEB34 (see legend of Fig.9), were used to PCR-amplify the exon 7 region of the α-subunit cDNA to generate a 414 bp DNA fragment. The PCR product derived from αpSVL was differentiated from the mutation-carrying form derived from C745TαpSVL by digestion with *Msp*I. Two DNA fragments, 242 bp and 172 bp, were produced upon *Msp*I digestion of the PCR product of wild-type αpSVL; however, the destruction of this *Msp*I site by the C745T mutation resulted in an uncut 414 bp fragment from C745TαpSVL (Fig.9). This strategy was used to screen for C745TαpSVL in approximately 60 transformed *E.coli BMH*71-18 colonies divided into twenty-3-colony groups. Two 3-colony-groups were identified to contain DNA from C745TαpSVL (Fig.9) and two individual colonies containing C745TαpSVL were identified by further screening of the individual colonies within the two groups. Plasmid DNA was

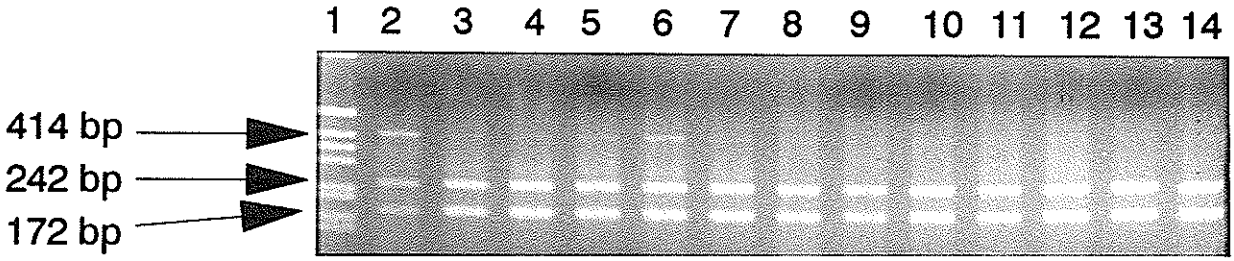



Fig.9. Identification of C745TαpSVL-containing bacterial colonies. *E. coli BMH*71-18, was used as a host for the transformation of C745TαpSVL plasmid DNA derived from the USE mutagenesis reaction (see section 2.12.1). Portions from three bacterial colonies were combined and used as a DNA source for PCR, with NEB29: 5'-TGTCATGGCGTACAATAAAT-3' and NEB34: 5'-GCAGGTGAAATCAACCTCAT-3' as primers. Ten µl of the PCR product was digested with *Msp*I and analysed by (2%) agarose gel electrophoresis. The C745T change destroyed an *Msp*I restriction site. A band corresponding to 414 bp (lanes 2 and 6) indicated the presence of C745TαpSVL. Lane 1, 1 kb ladder; lanes 2-14, each was a defined pool of three bacterial colonies.

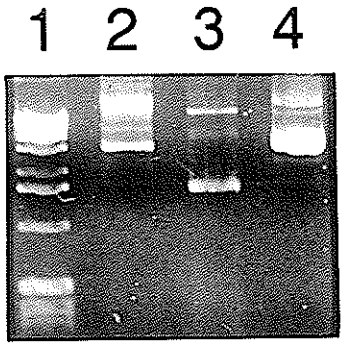
prepared in small-scale from one of the two C745TαpSVL-containing colonies and transformed into *E.coli* DH5α. The normal strain DH5α was used in the transformation to faithfully amplify the mutant-containing plasmid DNA. Forty transformed *E.coli* DH5α colonies were screened (as described above) and 15 of these were found to have only C745TαpSVL. Plasmid DNA from a C745TαpSVL-containing colony was prepared in large-scale and the whole cDNA insert was sequenced. The presence of C745T, and no additional base change, was observed.

To introduce the mutations G805A, G749A and C508T into αpSVL, *in vitro* oligonucleotide-directed mutagenesis was used. The detailed procedure is described in section 2.12.2. Briefly, the α-subunit cDNA was subcloned into $pBS^+$ to produce pHHEXA2 that was then transformed into *E.coli* JM109 for propagation and production of single-stranded pHHEXA2 DNA. To introduce a mutation, a mutagenic primer was annealed to the single-stranded pHHEXA2, extended with T7 polymerase and ligated. To amplify and select mutant pHHEXA2, the reaction product was separated by agarose gel electrophoresis, the DNA in the band corresponding to the newly-synthesized double-stranded pHHEXA2 (Fig.10a. lane 2) was purified, transformed into *E.coli* DH5α and mutant pHHEXA2-containing colonies were identified using PCR-based methods (see below). A mutant α-subunit cDNA derived from the mutant pHHEXA2 was then subcloned into pSVL to generate various mutant αpSVL vectors. The entire cDNA region was sequenced to confirm the presence of only the mutation of interest.

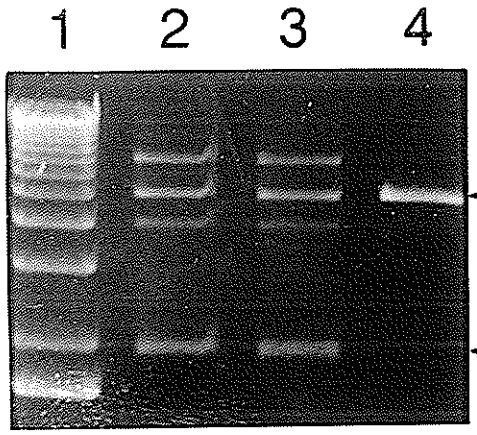
One example of the ligation products transformed to produce the mutant αpSVL constructs is shown in Fig.10b. The DNA band above that of the linear pSVL must be the

Fig.10. a. Detection of double-stranded DNA synthesized by oligonucleotide-directed mutagenesis. The reaction mix (10 µl) for oligonucleotide-directed mutagenesis of G805A (see section 2.12.2) was analysed by (1%) agarose gel electrophoresis (see section 2.2). Lane 1, 1 kb Ladder; lane 2, the reaction mixture; lane 3, single-stranded αpHHEXA2 DNA; lane 4, double-stranded αpHHEXA2 DNA. b. Analysis of Ligation Products. An aliquot (~100 ng) of a mutant α-subunit cDNA insert, generated by *Bam*H1 and *Xho*I digestion of a G805A-containing αpHHEXA2 and isolated using a GENECLEAN kit, was ligated with *Bam*H1/*Xho*I digested pSVL (~200 ng) in a 10 µl reaction mix using 1 U ligase (Gibco/BRL) at room temperature overnight. Five µl of the ligation product was analysed by (1%) agarose gel electrophoresis. Lane 1, 1 kb ladder; lanes 2 and 3, ligation reaction mix; lane 4, pSVL cut with *Bam*H1 and *Xho*I and ligated as a control.

a




b



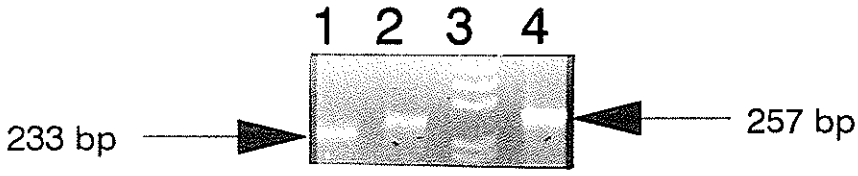
ligation product of interest. As expected, the pSVL ligation control vector that was restriction enzyme digested and dephosphorylated showed no DNA band above the level of the linear pSVL DNA.

To detect the G805A, G749A and C508T mutations, PCR-based strategies were used. The source of DNA for PCR was either transformed bacterial colonies or isolated plasmid DNA. A specific pair of primers was used to amplify the DNA region of interest that was used to detect each mutation. To detect the G805A mutation, the G805A-containing region was PCR-amplified using the primers NEB29 and WPG39 (see legend of Fig.11a for the sequence) to generate a 257 bp DNA fragment. The G805A change creates a *Sca*I site when the anti-sense primer WPG39, that contained a single base substitution and a single base deletion, is used in the PCR reaction. After a *Sca*I digestion, the 257 bp DNA fragment derived from the wild-type αpSVL remained the same, but that derived from G805AαpSVL was cut into two DNA fragments, 233 bp and 24 bp (Fig.11a). Eighteen colonies were screened for the presence of G805AαpSVL and 6 G805AαpSVL-containing colonies were identified.

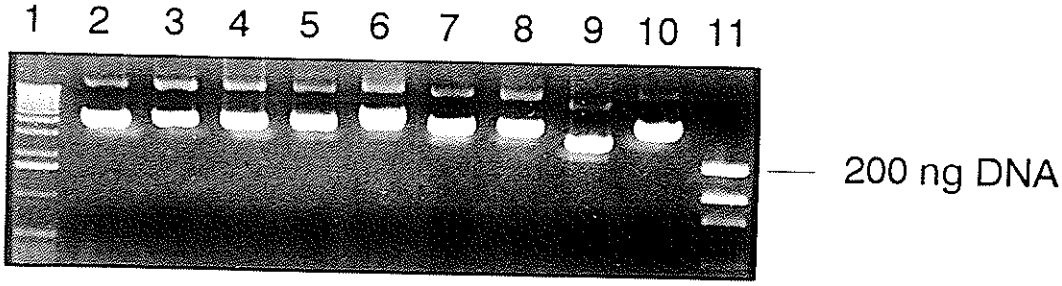
To detect the G749A change, the G749A-containing region was PCR-amplified using the primers NEB31: 5'-TACAACCCTGTCACCCACAT-3' and NEB34 (see legend to Fig.9 for sequence), resulting in a PCR product 305 bp in size. The 305 bp DNA fragment contained a new *EcoR*V site 72 bp from the 5' end of this fragment in the presence of G749A. After digestion with *EcoR*V, the 305 bp DNA fragment derived from αpSVL remained unchanged, but that derived from G749AαpSVL resulted in 233 bp and 72 bp DNA fragments (data not shown). Sixty colonies were screened for the presence of G749AαpSVL

Fig.11. a. Detection of the G805A mutation. The G805A-containing region was amplified by PCR using WPG39: 5'-CAAGGAGTCAGTAATCCAGAGTAC-3' and NEB29 (described in Fig.9 legend) as primers. Ten µl of the PCR products were digested with *Sca*I at 37°C for 2 hr and analysed by (2%) agarose gel electrophoresis. Lane 1, *Sca*I-digested PCR product derived from G805AαpSVL; lane 2, undigested PCR product derived from G805AαpSVL; lane 3, 1 kb Ladder; lane 4, *Sca*I-digested PCR product derived from αpSVL. The 24 bp DNA fragment had migrated off the bottom of the gel. b. Estimation of the DNA Concentration of Normal and Mutant Expression Vectors. An equal amount (0.5 µg, determined by reading absorbance at 260 nm, see section 2.1.0) of each expression vector was analysed by (1%) agarose gel electrophoresis. Lane 1, 1 kb Ladder; lane 2, αpSVL; lane 3, C739TαpSVL; lane 4, C745TαpSVL; lane 5, G805AαpSVL; lane 6, G749AαpSVL; lane 7, C508TαpSVL; lane 9, pCD43; lane 10, pRc/CMV-ß-gal; and lane 11, MASS Ladder DNA. The concentration of the marker was based on the information provided by Gibco/BRL.

a




b



and 9 G749AαpSVL-containing colonies were identified. To detect the C508T mutation, the C508T-containing region was PCR-amplified using the primers NEB25: 5'-TCTGGAGACTTTTAGCCAGC-3' and NEB30: 5'-AGTGAAGCTCTCATATGGGA-3' to produce a 238 bp DNA fragment. The 238 bp PCR product was tested for the C508T mutation that destroyed a *Hpa*II site 95 nucleotides from the 5' end of the fragment. Upon digestion with *Hpa*II, the 238 bp DNA fragment generated from wild-type αpSVL was cut into 143 bp and 95 bp DNA fragments, however, that from C508TαpSVL remained the same (data not shown). Sixty colonies were screened for the presence of C508TαpSVL and 11 C508TαpSVL-containing colonies were identified.

The major advantage of using the *in vitro* oligonucleotide-directed mutagenesis method over the USE mutagenesis method was that with the former method we were able to introduce mutations with high efficiency (greater than 20%), compared with an efficiency of about 2% with the USE method. In the former method, the time lapsed during removal of the DNA band corresponding to double-stranded DNA from the agarose gel should be minimized to avoid DNA damage by the UV light. The disadvantages of this method compared to USE mutagenesis were the additional required steps. These included subcloning the cDNA fragment into a pBS plasmid, the preparation of single-stranded DNA, and resubcloning the mutation-containing DNA into the αpSVL expression vector.

Once the different mutation-containing expression vectors were obtained, these plasmid vectors were prepared in large-scale for transfection. To prepare the DNA in large-scale for transfection, normal and mutant αpSVL DNAs were purified using Nucleobond-AX columns (see section 2.6.0). The purity of the DNA was assessed by agarose gel

electrophoresis and the DNA samples were quantitated by absorbance at 260 nm and by comparison with the DNA MASS Ladder as shown in Fig.11b. All of the vectors used for transfection were analyzed by (1%) agarose gel electrophoresis; they appeared to be of good quality (Fig.11b). The G749AαpSVL DNA in lane 6 of Fig.11b migrated slightly slower than the other normal and mutant αpSVL DNAs. This difference was not seen when the same DNA sample was analyzed on a freshly prepared agarose gel.

3.1.2 Effect of the benign mutations, R247W and R249W, on transiently expressed Hex A and Hex S.

**Effect of the Benign Mutations on Hex S Activity and the α-Subunit Protein Levels.** To determine the effects of the benign mutations on the activity of Hex S (αα) and/or the α-subunit protein level, the normal and mutant αpSVLs were transiently transfected into Cos-7 cells to express normal and mutant Hex S (αα). The mutant αpSVLs included C739TαpSVL, C745TαpSVL, G805AαpSVL, and C508TαpSVL, containing mutations associated with Hex A pseudodeficiency (2 vectors), adult-onset and infantile forms of $G_{M2}$ gangliosidosis, respectively. The resulting expressed Hex S activity was quantitated using 4-MUGS, a synthetic substrate specific to the α-subunit (Kresse *et al.*, 1981; Bayleran *et al.*, 1984). The protein levels of the expressed α-subunit were estimated using western blot analysis. Co-expression of ß-galactosidase, as measured using 4-MU-ß-gal as the substrate, from pRc/CMV-ß-gal was used as a measure of the transfection efficiency.

The specific activities of the expressed normal and mutant Hex S were normalized based on the expressed level of ß-galactosidase. Both normalized and non-normalized values

are presented in Table 2. For comparison, the Hex S activity expressed from wild-type αpSVL, after subtracting the Cos-7 cell background and normalization, was expressed as 100%. After the same calculation, the activities of the various mutant Hex S isoenzymes were converted to a percentage of the wild-type (Fig.12A).

The results showed that normal Hex S activity was expressed to a level more than 10 times that of the ß-hexosaminidase activity in the Cos-7 cells. However, the benign mutations, R247W and R249W, expressed Hex S activity just slightly more than four times higher than the endogenous activity in Cos-7 cells before normalization (Table 2). The Hex S activity expressed from the α-subunit cDNA containing the benign mutations was about 20-35% of the expressed normal Hex S activity (Fig.12A). The benign mutations clearly reduced the level of the Hex S activity compared to that of the wild type. This was consistent with the prediction that benign mutations were the cause of Hex A deficiency in enzyme-defined carriers and of Hex A pseudodeficiency when in combination with a second disease-causing allele.

Compared to the level of Hex S activity expressed by the vector carrying disease-causing mutations, the expressed benign mutation-containing Hex S activity was substantially higher (Fig.12A). This is consistent with the absence of clinical manifestations in Hex A pseudodeficient subjects, which suggests that these individuals have enough residual Hex A activity to adequately hydrolyse $G_{M2}$ ganglioside. The G269S substitution, associated with adult-onset $G_{M2}$ gangliosidosis (Navon and Proia, 1989; Paw *et al.*, 1989), expressed Hex S activity just above the background in Cos-7 cells and less than 5% of the normal. This was consistent with previous observations of the level of expressed Hex S activity containing

Table 2. Normal and mutant Hex S activities[a] expressed in Cos-7 cells.

| | αpSVL | | C739T αpSVL | | C745T αpSVL | | G805A αpSVL | | C508T αpSVL | | Mock |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | NN | N | NN | N | NN | N | NN | N | NN | N | |
| I | 735 | 680 | 390 | 225 | 280 | 285 | 110 | 65 | 68 | 44 | 60 |
| II | 755 | 715 | 535 | 240 | 360 | 390 | 160 | 50 | 70 | 35 | 75 |
| III | 840 | 820 | 335 | 265 | 300 | 300 | 105 | 75 | 65 | 65 | 90 |
| Mean | 776 | 738 | 420 | 243 | 313 | 325 | 125 | 63 | 68 | 48 | 75 |
| ± SD | ± 55 | ± 72 | ± 103 | ± 20 | ± 41 | ± 56 | ± 30 | ± 12 | ± 3 | ± 15 | ± 15 |

a: The activity is expressed as nmol 4-MUGS hydrolysed/hr/mg protein.

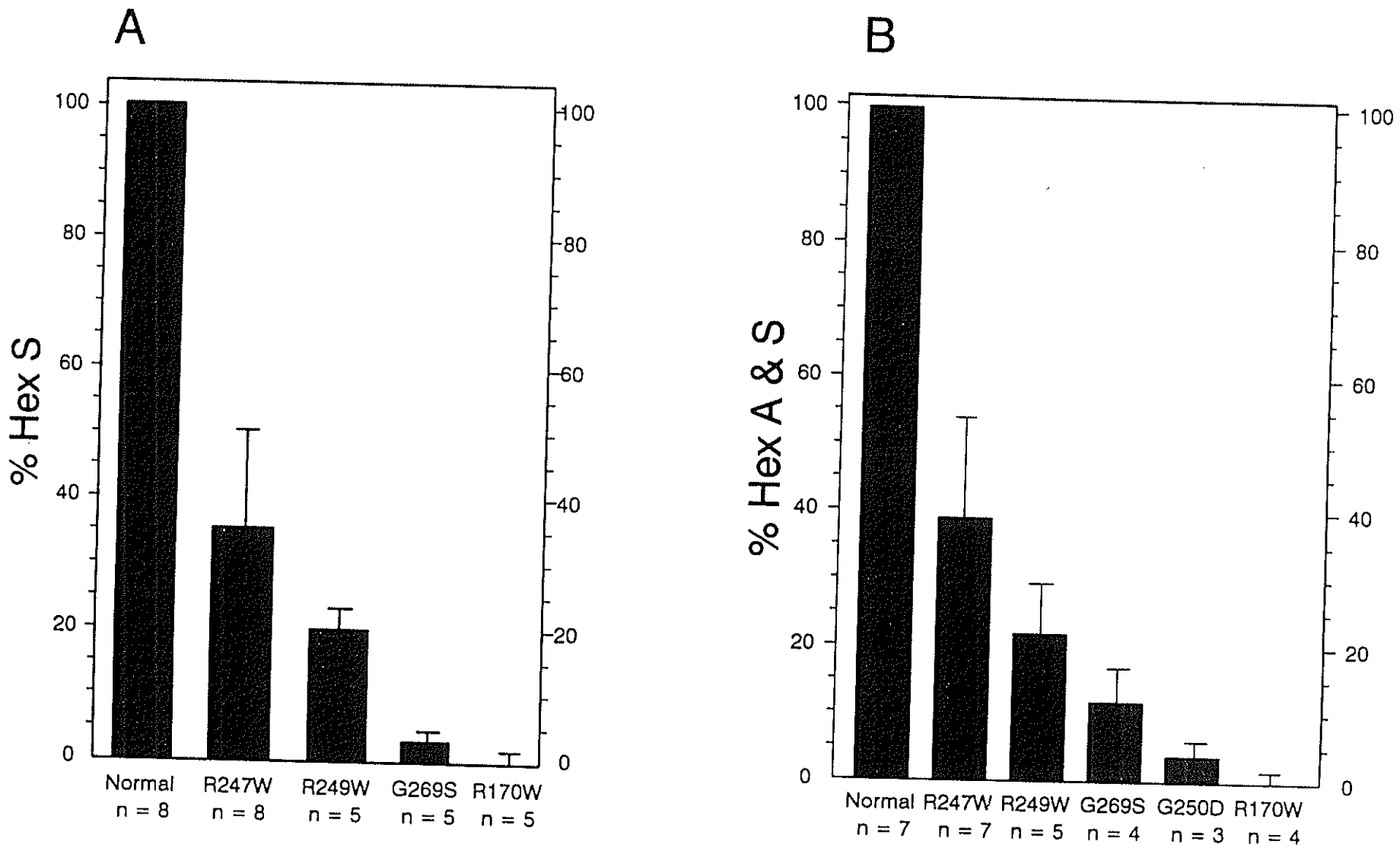
N: Activities were normalized by subtracting the endogenous ß-hexosaminidase activity in Cos-7 cells and multiplying the correction factor derived from the level of co-expressed ß-galactosidase activity.

NN: Non-normalized values.

I, II, III represent three sets of experiments. Each experiments included duplicate transfections; the assays for each transfection were done in duplicate. Additional data were included in the calculation of the values for figure 12A.

Mock: no exogenous DNA was transfected.

Fig.12. (Panel A) Expression of Hex S ($\alpha\alpha$) and (Panel B) Hex A & S activities in Cos-7 cells. Both Hex S and Hex A & S were expressed in Cos-7 cells. Normal and mutant αpSVL (6.5 μg) were transfected alone for Hex S expression or together with pCD43 (2.3 μg) for Hex A & S expression. The expressed Hex S and Hex A & S activities were determined using the synthetic substrate 4-MUGS. The specific activities were normalized after the subtraction of the mock-transfected background levels. Normalization was based on the co-expressed ß-galactosidase activity. The average wild type specific activity was defined as 100% and the mutant levels are shown as a percent of the wild type level. The number of experiments (n) represented by each bar is shown. Each experiment contained duplicate transfections. The error bars represent the sample standard deviation.

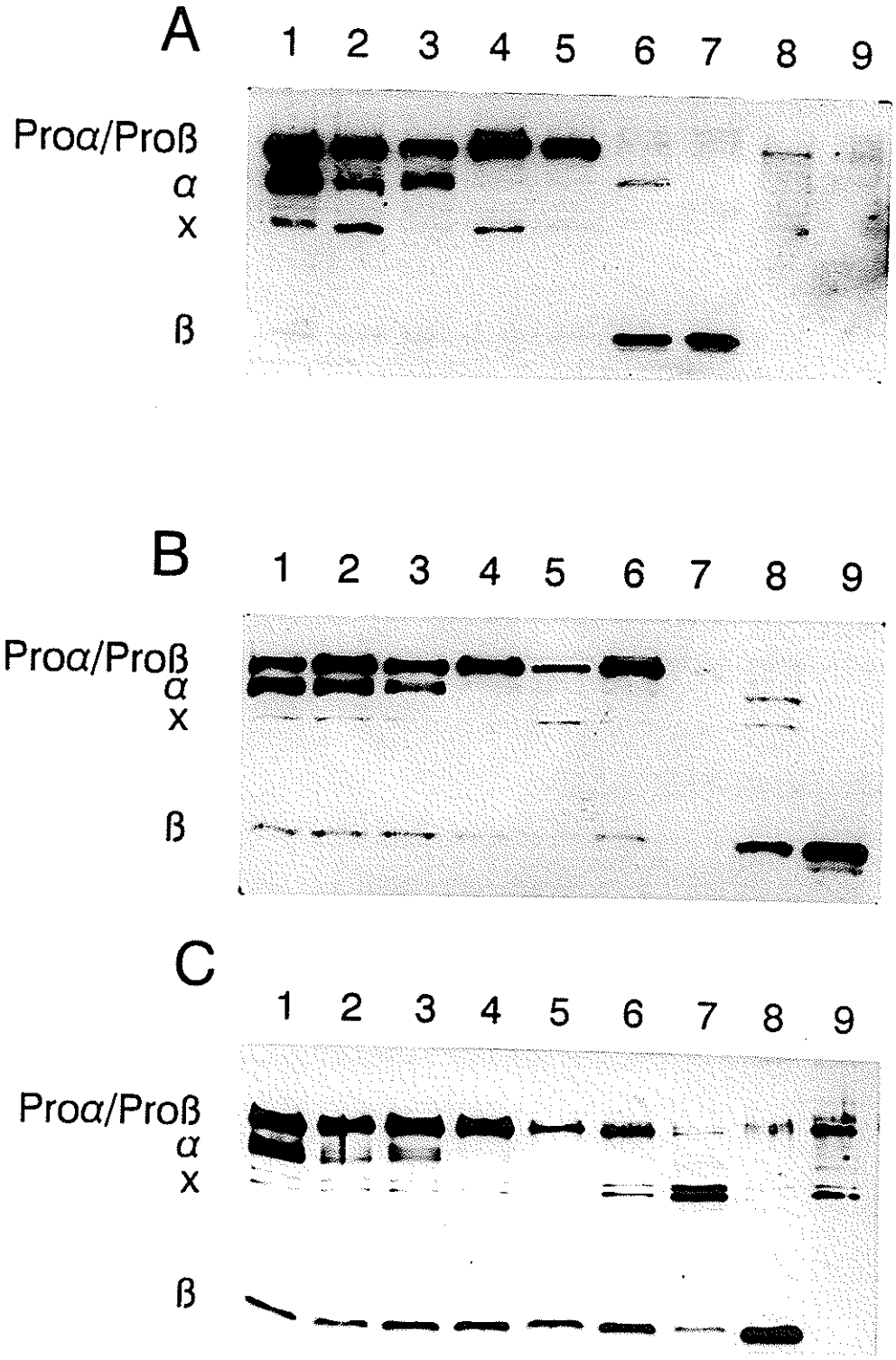

A
% Hex S
100
80
60
40
20
0
Normal
n = 8
R247W
n = 8
R249W
n = 5
G269S
n = 5
R170W
n = 5
B
% Hex A & S
100
80
60
40
20
0
Normal
n = 7
R247W
n = 7
R249W
n = 5
G269S
n = 4
G250D
n = 3
R170W
n = 4

G269S mutation (Navon and Proia, 1989; Brown and Mahuran, 1993) and also corresponded to the clinical late-onset phenotype. The vector carrying the Tay-Sachs disease mutation C508T(R170W) in the α-subunit, as we expected, expressed no Hex S activity.

The expression of Hex S in Cos-7 cells also allowed some interpretations about how the benign mutations affect the α-subunit to be made. The expression of a substantial level of Hex S activity indicated that a considerable level of α-subunit must be synthesized and processed. This was confirmed by western blot analysis which showed there was a considerable amount of the precursor and mature forms of the α-subunit protein harbouring the benign R247W and R249W mutations (Fig.13A), suggesting they did not affect synthesis and processing of the α-subunit. However, the level of the mature α-subunit protein containing the benign mutations appeared to be less than 40% of the wild type level (Fig.13A), consistent with its reduced enzyme activity. In comparison, no detectable mature form of the α-subunit corresponding to the G269S-containing Hex S and R170W-containing Hex S was observed on the western blot (Fig.13A), even though the precursor α-subunit levels appeared similar to that of the normal. To substantiate the identity of the bands detected on the western blot, a series of controls were included. Normal fibroblasts showed mature forms of the α- and ß-subunits (Fig.13A, lane 6); Tay-Sachs disease fibroblasts did not show the α-subunit, but showed the mature form of the ß-subunit (Fig.13A, lane 7); Sandhoff disease fibroblasts did not have ß-subunit, but did contain precursor α-subunit (Fig.13A, lane 8). The transfected Cos-7 cells did not show the ß-subunit because only the α-subunit cDNA was transfected (Fig.13A, lanes 1-5).

Effect of Benign Mutations on Hex A & S Activity and the Level of the α-Subunit

Fig.13. Levels of expressed α-subunit protein containing the benign and disease-causing mutations. The α-subunit cDNA, and its various mutant derivatives, were transfected alone to express Hex S (Panel A) or together with the ß-subunit to express Hex A & S (Panels B and C). The amount of protein loaded for each sample within a panel (A and B was 20-30 μg, C was 5-10 μg) was normalized to the co-expressed ß-galactosidase activity, with the exception of the Mock (25-30 μg) and fibroblast (30-50 μg) samples. The α- and ß-subunits of ß-hexosaminidase were detected with a polyclonal anti-Hex A antibody. (PANEL A) Lane 1, αpSVL (normal); lane 2, C739TαpSVL (benign); lane 3, C745TαpSVL (benign); lane 4, G805AαpSVL (adult-onset); lane 5, C508TαpSVL (infantile); lane 6, MCH065 fibroblast (normal); lane 7, WG1881 fibroblast (Tay-Sachs disease); lane 8, GM00294 fibroblast (Sandhoff disease); lane 9, mock transfected Cos-7 cells (background). (PANEL B) Lane 1, αpSVL (normal); lane 2, C739TαpSVL (benign); lane 3, C745TαpSVL (benign); lane 4, G805AαpSVL (adult-onset); lane 5, G749AαpSVL (juvenile); lane 6, C508TαpSVL (infantile); lane 7, Mock-transfected Cos-7 (Background); lane 8, WP09 fibroblast (normal), lane 9, 1492 fibroblast (Tay-Sachs disease). (PANEL C) lanes 1 to 7 are the same as in panel B, lane 8, WG1881 fibroblast (Tay-Sachs disease), lane 9, GM00294 fibroblast (Sandhoff disease). An "x" indicates cross-reacting protein of unknown identity.


A
1 2 3 4 5 6 7 8 9
Proα/Proß
α
x
ß
B
1 2 3 4 5 6 7 8 9
Proα/Proß
α
x
ß
C
1 2 3 4 5 6 7 8 9
Proα/Proß
α
x
ß

Protein. To examine the effects of the benign mutations, R247W and R249W, on the Hex A & S activity and the α-subunit protein level, Hex A & S were transiently expressed by introducing pCD43, together with wild-type αpSVL or its mutant variants, into Cos-7 cells. These mutant αpSVLs included C739TαpSVL, C745TαpSVL, G805AαpSVL, G749ATαpSVL, and C508TαpSVL, associated with Hex A pseudodeficiency (2 vectors), adult-onset, juvenile and infantile forms of $G_{M2}$ gangliosidosis, respectively. Hex A & S activity was measured using 4-MUGS as the substrate and the levels of the α-subunit protein were estimated by western blot analysis. ß-Galactosidase was co-expressed to estimate the transfection efficiency.

The specific activities of the expressed normal and mutant Hex A & S were normalized according to the expressed level of ß-galactosidase activity, and the activities before and after the normalization are presented in Table 3. For comparison, the expressed wild-type Hex A & S activity, after subtracting the Cos-7 cell background and normalization(see section 2.17), was taken as 100% and the activities of the various mutant Hex A & S, after similar calculations, were expressed as a percentage of the normal (Fig.12B).

The normal Hex A & S activity expressed by co-transfecting the α- and ß-subunit cDNAs was more than 10 times higher than the endogenous ß-hexosaminidase activity in Cos-7 cells. The Hex A & S containing the benign mutations appeared about 4-5 times higher than the activity in Cos-7 cells before normalization (Table 3). After normalization, the benign mutations, R247W and R249W, reduced the Hex A & S activity to 20-40% of that of the normal (Fig.12B). This once again demonstrated the capacity of the benign mutations

Table 3. Normal and mutant Hex A & S activities[a] expressed in Cos-7 cells

| | αpSVL | | C739T αpSVL | | C745T αpSVL | | G805A αpSVL | | G749A αpSVL | | C508T αpSVL | | Mock |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | NN | N | NN | N | NN | N | NN | N | NN | N | NN | N | |
| I | 1535 | 1385 | 760 | 820 | 600 | 560 | 325 | 260 | 160 | 0 | 165 | 0 | 160 |
| II | 1355 | 1390 | 600 | 690 | 485 | 400 | 245 | 130 | 125 | 25 | 105 | 13 | 95 |
| III | 1385 | 1425 | 550 | 630 | 515 | 335 | 275 | 115 | 165 | 35 | 125 | 17 | 117 |
| Mean | 1425 | 1400 | 637 | 713 | 533 | 432 | 282 | 169 | 150 | 20 | 132 | 10 | 124 |
| ± SD | ± 96 | ± 22 | ± 109 | ± 153 | ± 59 | ± 115 | ± 40 | ± 79 | ± 22 | ± 18 | ± 30 | ± 8 | ± 33 |

a: The activity is expressed as nmol 4-MUGS hydrolysed/hr/mg protein.

N: Activities were normalized by subtracting the endogenous ß-hexosaminidase activity in Cos-7 cells and multiplying by the correction factor derived from the level of co-expressed ß-galactosidase activity.

NN: Non-normalized values.

I, II, III represent three sets of experiments. Each experiments included duplicate transfections; the assays for each transfection were done in duplicate. Additional data were included in the calculation of the values for figure 12B.

Mock: no exogenous DNA was transfected.

to cause Hex A deficiency. The benign mutation-containing Hex S (Fig.12A) and Hex A & S (Fig.12B) showed a similar percent (20-40%) of the normal activity levels suggesting that the ß-subunit had no effect on the benign mutant-containing α-subunit through dimerization.

The activity levels of the expressed benign mutation-containing Hex A & S were higher than that of the disease-causing mutations (Fig.12B). The adult-onset disease mutation G269S produced about 12% of the normal, significantly less than either of the benign mutations. The higher activity level of G269S mutation-containing Hex A & S than that of Hex S (less than 5%) with the same mutation might be that the ß-subunit stabilized the α-subunit containing G269S mutation through dimerization, as proposed by Brown and Mahuran (1993). The G250D mutation showed Hex A & S activity less than 4% of the normal level matching its association with a juvenile onset clinical phenotype (Hechtman *et al.*, 1989). The Tay-Sachs disease mutation R170W, as expected, did not express Hex A & S activity. The expression of Hex A & S allowed mutations associated with different levels of residual ß-hexosaminidase activity to be more clearly differentiated.

The protein levels of the α-subunit in Hex A & S were also decreased by the benign mutations, R247W and R249W (Fig.13B and C). The levels of the precursor α-subunit containing the benign mutations appeared at the same level as the normal, however the levels of the mutant mature α-subunit were significantly less than that of the normal. Western blots from two separate experiments are presented to show the degree of variation between the experiments.

All of the disease-causing mutations showed the absence of the mature α-subunit and a significant amount of the precursor form, indicating that these mutations did not affect the

synthesis of the α-subunit (Fig.13B and C). The G269S mutation was shown to affect the stability of the α-subunit and indirectly affect the association of the α-subunit with the ß-subunit (Brown and Mahuran, 1993); this residue G269 was predicted to locate on the surface of the α-subunit (Tews *et al.*, 1996). The G250D mutation, associated with the juvenile-onset form of $G_{M2}$ gangliosidosis, was previously shown to affect the phosphorylation, secretion and conversion from the precursor to the mature form of the α-subunit (Trop *et al.*, 1992; Hechtman *et al.*, 1989). This residue, G250, was predicted to locate in a hydrophobic domain (Trop *et al.*, 1992) or in the core of the α-subunit (Tews *et al.*, 1996). The Tay-Sachs disease mutation R170W had not been previously characterized. These results suggested that this mutation might affect the processing of the α-subunit rather than the enzyme function. The controls, as shown in Fig.13B and C, were designed for the same purpose as those used for the study of Hex S expression and described earlier (Fig.13A). The Hex A & S expression results showed that the benign mutations could be differentiated from the normal and the disease-causing mutations by both the levels of activity and the mature α-subunit protein (Fig.12B and Fig.13B and C). This was also apparent in the Hex S expression analysis (Fig.12A and Fig.13A), although the disease-causing mutations were all similar in this study. The differences between the benign mutations and the disease-causing mutations appeared more obvious at the level of the mature α-subunit protein than at the activity level.

The western blot analysis showed the ratio of the protein levels of the mature α-subunit to the ß-subunit of the wild type was much higher in the transfected Cos-7 cells than that in the normal fibroblasts (Fig.13B and C, lanes 1 and 8). This supported the prediction that the α-subunit was over expressed, but it suggested that the ratio of the α- and ß-subunit

cDNA vectors, αpSVL and pCD43, had not been optimized adequately. This was confirmed by the isoenzyme separation (see section 3.3.0).

3.2 Effect of the benign mutation, R247W, on Hex A activity and the protein level of the α-subunit in hex a pseudodeficient fibroblasts.

Hex A from fibroblasts of pseudodeficient individuals was examined to confirm the previous findings of Thomas *et al.* (1982) and Grebner *et al.* (1986) and to provide a second characterized source for the isolation of mutant Hex A for analysis. The Hex A activity and the α-subunit protein from fibroblasts from two Hex A pseudodeficient subjects were compared with that of normal fibroblasts. The synthetic substrate, 4-MUGS, was used as the substrate to determine the ß-hexosaminidase activity and a western blot was used to examine the α-subunit protein level. Both lines of Hex A pseudodeficient fibroblasts, TC72 and GM04863 (Triggs-Raine *et al.*, 1992), produced about 36-41% of the normal Hex A activity (Table 4) and a comparable level of mature α-subunit protein (Fig.14). This is consistent with previous studies where pseudodeficient fibroblasts had Hex A activity that was 23-26% of the total ß-hexosaminidase activity compared to 49-65% Hex A for the normal fibroblasts (Thomas *et al.*, 1982). Leupeptin, a lysosomal protease inhibitor at 200 μM (Seglen, 1983), in the cell culture medium did not increase the percentage of Hex A activity (Table 4) or the level of mature α-subunit protein (Fig.14) in either the normal or mutant fibroblasts.

The results from the studies using the fibroblasts were consistent with the observations found using the Cos-7 expression system. Unfortunately, a fibroblast cell line carrying the benign mutation, R249W, could not be obtained and only the Hex A pseudodeficient

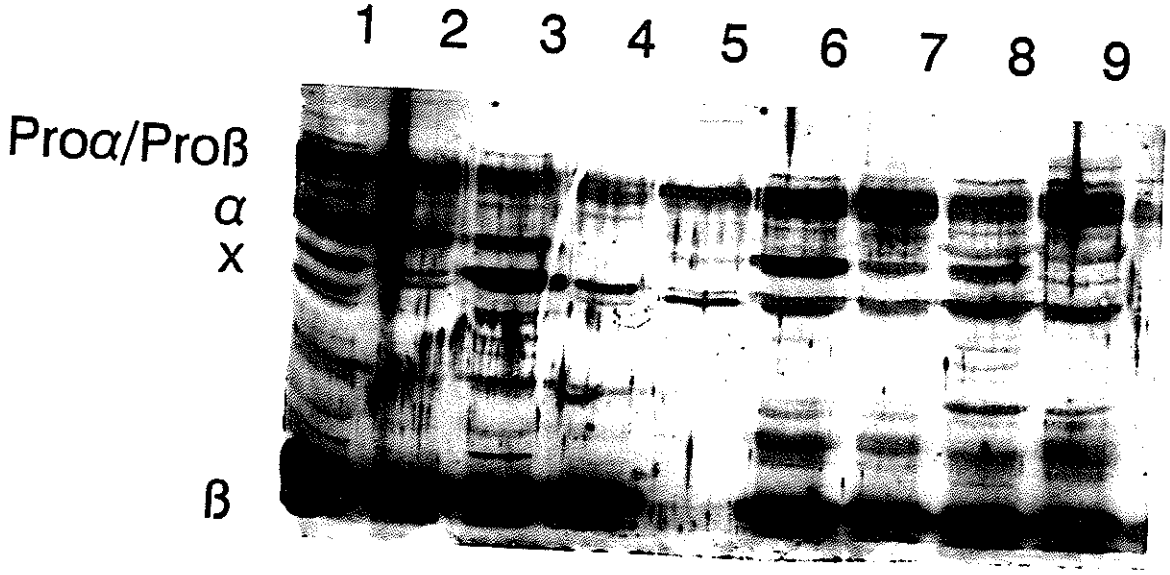



Fig.14. Levels of the α-subunit protein in Hex A pseudodeficient fibroblasts. Fibroblasts were grown to confluence in a 20 x 150 mm tissue culture dish, then subcultured in two 20 x 100 mm tissue culture dishes. After 48 hr of growth in α-MEM supplemented with antibiotics and 10% fetal bovine serum in the presence (lanes 6-9) or absence (lanes 1-5) of Leupeptin (200 μM), cell extracts were prepared. Cell extracts (30 μg protein) were separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane, and the ß-hexosaminidase proteins were detected with the anti-Hex A antibody. Lane 1, MCH65 (normal); lane 2, TC72 (benign); lane 3, GM04863 (benign); lane 4, WG1881 (Tay-Sachs disease); lane 5, GM00294 (Sandhoff disease); lane 6, MCH65 (normal); lane 7, TC72 (benign); lane 8, GM04863 (benign); lane 9, WG1881 (Tay-Sachs disease).

Table 4. ß-Hexosaminidase A Activity[a] in Cultured Fibroblasts.

| | MCH065 | TC72 | GM04863 | WG1881 | GM00294 |
|---|---|---|---|---|---|
| -leupeptin[b] | 332 ± 64 | 120 ± 16 | 137 ± 19 | 13 ± 3 | 43 ± 3 |
| %[c] | 100 % | 36 % | 41 % | 4 % | 13 % |
| +leupeptin[b] | 419 ± 52 | 141 ± 8 | 154 ± 5 | 15 ± 2 | 78 ± 5 |
| %[c] | 100 % | 34 % | 37 % | 4 % | 19 % |

[a]The activities were determined by incubating a 30 $\mu$l reaction mix containing 4 to 8 $\mu$g of cell extract protein and 20 $\mu$l of 4 mM 4-MUGS in citrate-phosphate buffer, 0.3% BSA, pH 4.4, for 120 min at 37°C. The activities are defined as nmol 4-MUGS hydrolysed/hr/mg protein. The data represent the average of four experiments using three plates of each fibroblast cell line. The enzyme assay on each plate was carried out in triplicate.

[b]+/- leupeptin indicates the presence (+) or absence (-) of 200 $\mu$M leupeptin in the cell culture medium for 48 hr.

[c]Hex A activities of the Hex A pseudodeficient cell lines, TC72 and GM04863, a Tay-Sachs disease cell line WG1881, and a Sandhoff disease cell line, GM00294 are presented as a percentage of the activity of a normal fibroblast cell line MCH065 (100%).

fibroblast cell lines carrying the benign mutation, R247W, were used in this study. Consequently, both Cos-7 expressed mutant Hex A and fibroblast Hex A were used for the analysis of the effects of the benign mutations on Hex A.

3.3 Characterization of Hex A harbouring benign mutations in the α-subunit.

The results from these studies and others (Thomas *et al.*, 1982; Grebner *et al.*, 1986; Triggs-Raine *et al.*, 1992) showed that Hex A harbouring a benign mutation in the α-subunit possessed activity toward the natural and synthetic substrates. However, the levels of activity measured with $G_{M2}$ ganglioside were consistent with a normal enzyme while those with the synthetic substrate were reduced. This suggested that these may be a problem in the recognition of the synthetic substrate. Our own studies suggested that the loss of activity toward the synthetic substrate reflected a decrease in the protein level. To assess these possibilities more fully, we decided to study the characteristics of Hex A harbouring the benign mutation. Hex A was initially separated from Hex B and Hex S to avoid their interference because these isoenzymes can also hydrolyse the synthetic substrates, 4-MUG or 4-MUGS. The isolation of Hex A from the transfected Cos-7 cell extracts was essential because it became apparent that the level of Hex S expressed in the Cos-7 cells was very high in comparison to Hex A (see below).

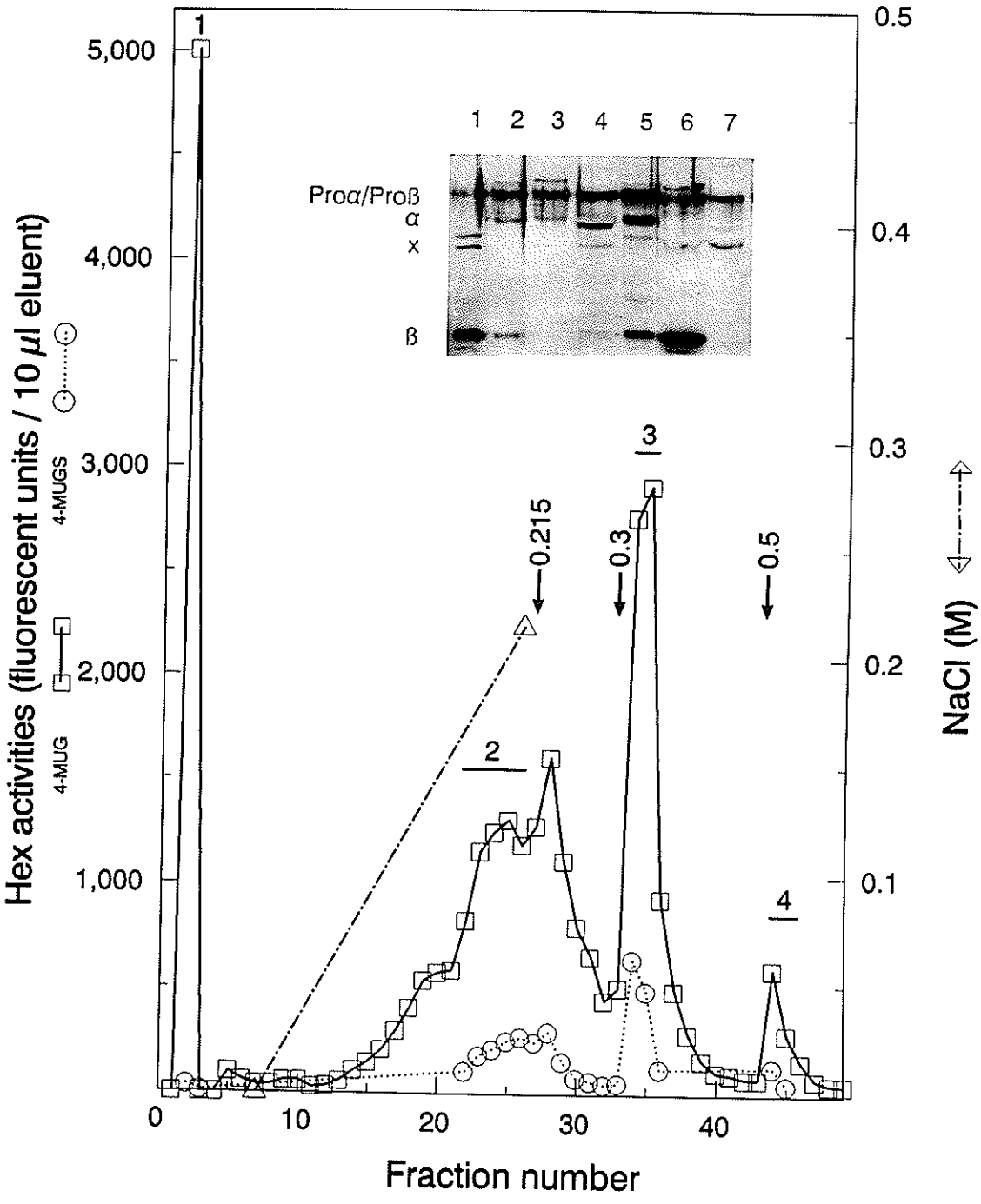
3.3.0 Separation of Hex A from Hex B and Hex S.

To separate Hex A from Hex B and Hex S, a protocol was developed based on the published methods by Mahuran *et al.* (1985) and Emiliani *et al.* (1990). In this protocol (see

section 2.18), a DEAE-cellulose column with a 1.5-3 ml bed volume was used to separate between 1-5 mg protein. The column was loaded with protein extract from Cos-7 cells transfected with the α- and ß-subunit cDNAs, and eluted with a combination of 10 mM phosphate buffer, pH 6.0, followed by a continuous and then discontinuous NaCl gradient (Fig.15). The ß-hexosaminidase activity was measured using both 4-MUG and 4-MUGS as substrates. It was found that the 0-0.215 M NaCl gradient, followed by 0.215 M NaCl, eluted almost all of the Hex A from the column, and 0.3 M NaCl eluted all the Hex S (Fig.15). The activity peaks corresponding to Hex A and Hex S possessed activities toward both synthetic substrates 4-MUG and 4-MUGS. The Hex B peak had activity only towards 4-MUG.

The identity of the eluted activity peaks was confirmed by western blot using anti-human Hex A as the primary antibody. The ß-subunit, and no mature α-subunit, was found in the void volume fractions (Hex B fractions), while both α- and ß-subunits were found in the fractions eluted with the 0-0.215 M NaCl gradient (Hex A fractions). The α-subunit, but no ß-subunit was found in the 0.3 M NaCl-eluted Hex S fraction, and a small amount of both the α- and ß-subunits were found in the 0.5 M NaCl-eluted fraction (Fig.15 insert figure). Even though the western blot analysis did not differentiate the α-subunit precursor from the ß-subunit precursor, signals from the mature α- and ß-subunit were clearly detected. In addition to the activity peaks corresponding to Hex B, A and S, an activity of unknown identity was found to be eluted with 0.5 M NaCl (Fig.15). Its biological function is unknown, but it apparently contains both the α- and ß-subunit. A similar activity peak eluted with 1 M NaCl was also previously observed (Emiliani *et al.*, 1990).

Fig.15. Separation of the ß-Hexosaminidase isoenzyme forms, Hex B, Hex A, and Hex S by anion exchange chromatography. Cell extract (1.7 mg protein) prepared from α-/ß-subunit cDNA co-transfected Cos-7 cells was loaded onto a DEAE-cellulose column (1.2 ml bed volume), that had been equlibrated with 10 ml of 10 mM phosphate buffer, pH 6.0, and the column was eluted with a linear and then step gradient of NaCl extending to 0.5 M NaCl (see section 2.18). The linear NaCl gradient is shown as Δ- · -Δ; Start points for the steps of the gradient are denoted by ↓, the number on the top of the arrow indicates the molar concentration of NaCl. Column fractions (1 ml each) representing each peak of activity (1 to 4) were pooled and concentrated, ~20 μg of fractions 1 and 2, and ~40 μg of fractions 3 and 4, were separated by SDS-PAGE followed by western blot analysis to determine the identity of the peaks using the anti-Hex A antibody. In the inserted box, lane 1, no NaCl (Hex B); lane 2, 0-0.215 M NaCl (Hex A); lane 3, 0.3 M NaCl (Hex S); lane 4, 0.5 M NaCl (Hex A-related; function unknown); lane 5, Extract of Cos-7 cells transfected with pCD43/αpSVL (normal); lane 6, WG1881 fibroblast (Tay-Sachs disease), lane 7, GM00294 fibroblast (Sandhoff disease).

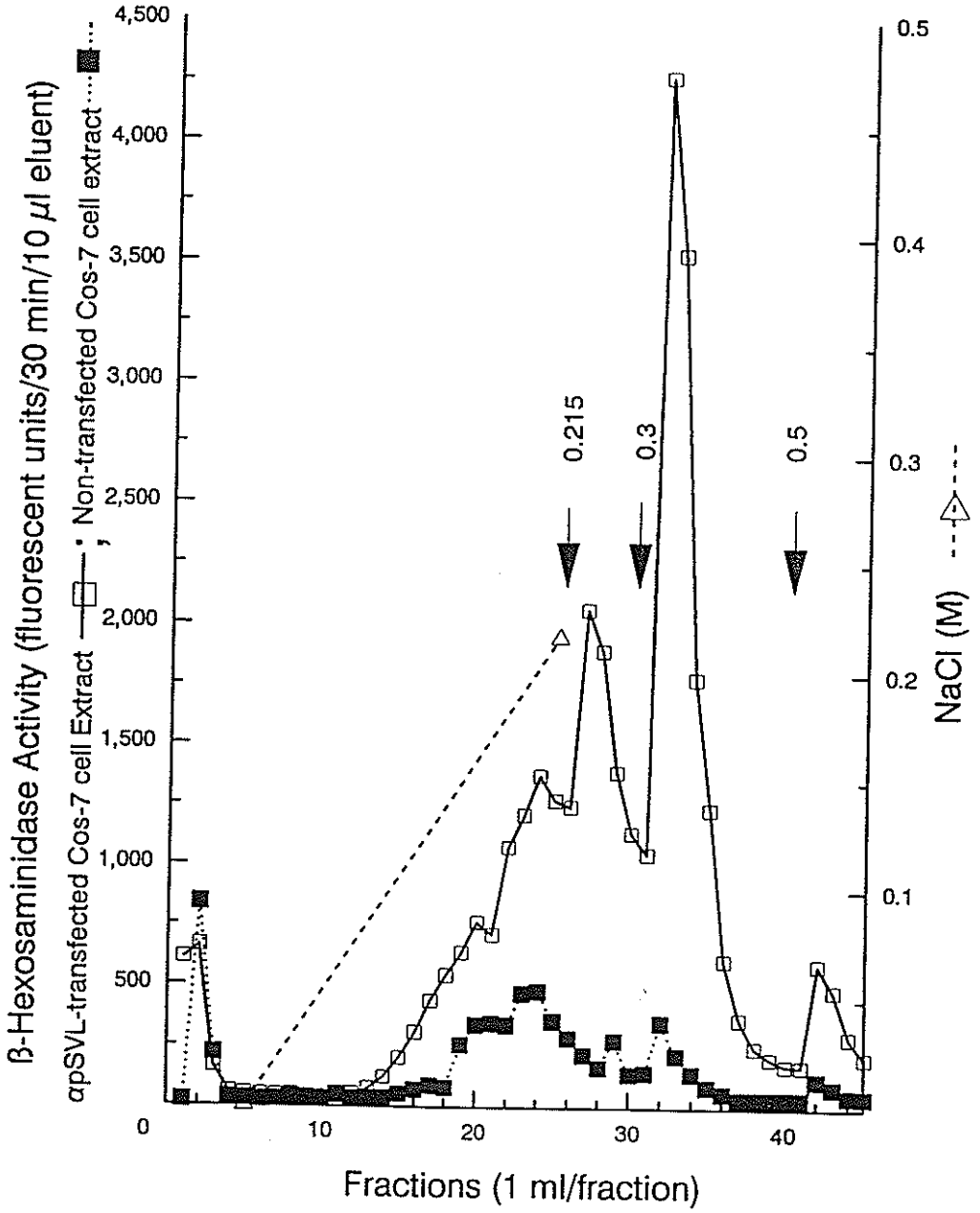

Hex activities (fluorescent units / 10 µl eluent)
4-MUG
4-MUGS
NaCl (M)
Fraction number
5,000
4,000
3,000
2,000
1,000
0
10
20
30
40
0.5
0.4
0.3
0.2
0.1
1
2
3
4
0.215
0.3
0.5
1 2 3 4 5 6 7
Proα/Proß
α
x
ß

Using the protocol described above for the separation of the different ß-hexosaminidase isoenzymes, the cell extracts of Cos-7 cells non-transfected and transfected with wild-type αpSVL to express Hex S were also analyzed. Once again, the separated ß-hexosaminidase activities were assayed using 4-MUG as the substrate. The ß-hexosaminidase activities from the non-transfected and transfected Cos-7 cells were normalized based on the level of loaded protein. The activity profile after the normalization is shown in Fig.16. The three ß-hexosaminidase isoenzyme activities, Hex B, Hex A, and Hex S were observed from both non-transfected Cos-7 cells and αpSVL-transfected Cos-7 cells. The levels of Hex A and Hex S activity from non-transfected Cos-7 cells appeared similar, although there was much less Hex S than Hex A activity; both were lower than the Hex B level. The ß-hexosaminidase activity profile of the αpSVL-transfected Cos-7 cells exhibited a predominant Hex S activity peak, however, a considerable level of Hex A activity, that was much higher than the basal Hex A activity, was also present. We speculate that Hex A is a dimer formed from the α-subunit encoded by the transfected αpSVL and the endogenous ß-subunit of the Cos-7 cells. The activity of unknown identity was also eluted by 0.5 M NaCl from cells transfected solely with αpSVL.

3.3.1 Effect of the benign mutations on the heat sensitivity of Hex A & S *in vitro*.

To determine if the benign mutations result in a heat-labile form of Hex A, cell extracts from normal (MCH065) and Hex A pseudodeficient (TC72) fibroblasts, or isolated Hex A from Cos-7 cells transfected with pCD43/αpSVL, pCD43/C739TαpSVL, pCD43/C745TαpSVL, or pCD43/G805AαpSVL were treated at 45°C and 50.1°C for

Fig.16. ß-Hexosaminidase activity profile from α-subunit cDNA transfected and non-transfected Cos-7 cells. Cell extracts from non-transfected (2.85 mg protein) and αpSVL transfected (5.04 mg protein) Cos-7 cells were separately loaded onto a DEAE-cellulose column (~2.5 ml bed volume) that had been equilibrated with 10 ml of 10 mM phosphate buffer, pH 6.0. The column was eluted with a linear and step gradient of NaCl (see section 2.18). ß-Hexosaminidase activity was determined by incubating 20 µl 3 mM 4-MUG in citrate-phosphate buffer, pH4.4, and 10 µl of the each column fraction at 37°C for 30 min. The activity was calculated and expressed as fluorescent units/30 min/10 µl eluent. The activities in the column fractions derived from the αpSVL-transfected Cos-7 cell extract were normalized based on the protein level of the loaded cell extract. The activities from the transfected Cos-7 cell extracts were corrected using $2.85 \div 5.04 = 0.565$ as the correction factor. Profiles of the normalized ß-hexosaminidase activities in the fractions from both the non-transfected and transfected Cos-7 cells are presented.


β-Hexosaminidase Activity (fluorescent units/30 min/10 μl eluent)
αpSVL-transfected Cos-7 cell Extract
Non-transfected Cos-7 cell extract
4,500
4,000
3,500
3,000
2,500
2,000
1,500
1,000
500
0
0.5
0.4
0.3
0.2
0.1
NaCl (M)
0.215
0.3
0.5
10
20
30
40
Fractions (1 ml/fraction)

intervals up to 1.5 hr or 2 hr, respectively. The remaining Hex A & S activities were measured at 37°C using 4-MUGS as the substrate. The activity in non-heated samples was taken as 100% and the activities in the heat-treated samples were converted into a percentage of the non-heated activity. The results showed that the activity of Hex A harbouring a benign mutation, R247W or R249W, in the α-subunit was decreased at a rate similar to that of the normal Hex A by the heat treatment at 45°C (Fig.17) and 50.1°C (Fig.18), but at a significantly slower rate than that of Hex A containing the adult-onset mutation G269S (Fig.17). The heat treatment of the cell extracts from normal fibroblasts and the fibroblasts containing the benign mutation R247W at 50.1°C showed similar results (Fig.19) to that from the transfected Cos-7 cells (Fig.18).

3.3.2 Determination of the pH optimum of the expressed Hex A & S with benign mutations.

The optimal pH for the hydrolysis of the synthetic substrate, 4-MUGS, by Hex A in the cell extracts from normal (MCH065) and Hex A pseudodeficient (TC72) fibroblasts (Fig.20) and Cos-7 cells transfected with pCD43/αpSVL or pCD43/C739TαpSVL or pCD43/C745TαpSVL (Fig.21), was 4.0 to 4.4. The Hex A activity in the transfected Cos-7 cells was higher than that in the fibroblasts, corresponding to the observations in previous experiments (Table 3 and Table 4). Each set of results were derived from two separate experiments.

3.3.3 $K_m$ and $V_{max}$ of Hex A harbouring a benign mutation in the α-subunit.

To determine the $K_m$ and $V_{max}$ of normal and mutant Hex A, Hex A was isolated from

Fig.17. Heat (45°C) sensitivity of Hex A & S expressed in Cos-7 cells. Approximately 50 μg of Hex A isolated from the cell extracts from pCD43/αpSVL, pCD43/C739TαpSVL, pD43/C745TαpSVL and pCD43/G805AαpSVL transfected Cos-7 cells, was treated at 45°C in 150 μl of citrate-phosphate buffer, pH4.4, in the presence of 0.3% BSA. Aliquots (10 μl) were taken at 0, 15, 30, 60 and 90 minutes of incubation and used to determine Hex A & S activity at 37°C using 4-MUGS as the substrate. The activities of the 0 minute incubation were defined as 100% and the subsequent values are expressed as a percent of the 0-minute-incubation activity. The data were derived from two experiments with duplicate samples at each point. Assays for Hex A & S activity were done in duplicate for each sample. The bar represents the sample standard deviation of the activity in these samples.

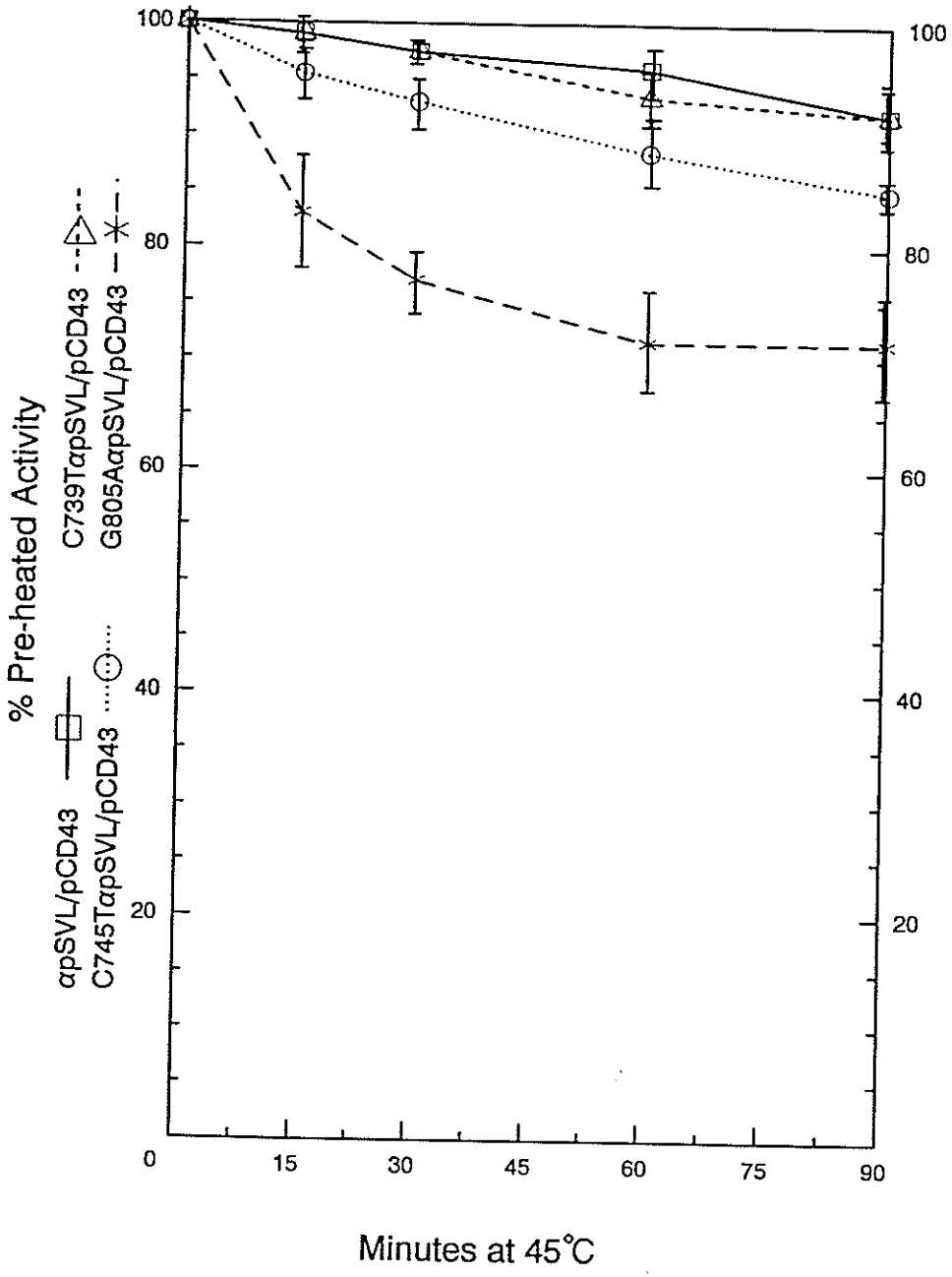

% Pre-heated Activity
αpSVL/pCD43
C745TαpSVL/pCD43
C739TαpSVL/pCD43
G805AαpSVL/pCD43
100
80
60
40
20
0
15
30
45
60
75
90
Minutes at 45°C

Fig.18. Heat (50.1°C) sensitivity of Hex A & S expressed in Cos-7 cells. Approximately 100 μg of protein from pCD43/αpSVL, pCD43/C739TαpSVL, and pCD43/C745TαpSVL transfected Cos-7 cell extracts were treated at 50.1°C in 150 μl of citrate-phosphate buffer, pH4.4, in the presence of 0.3% BSA. Aliquots (10 μl) were taken at 0, 15, 30, 60, 90, and 120 minutes of incubation and used to determine Hex A & S activity at 37°C using 4-MUGS as the substrate. The activities of the 0 minute incubation were defined as 100% and the subsequent values were expressed as a percent of the 0-minute-incubation activity. The data were derived from three experiments with duplicate samples at each point. Assays for Hex A & S activity were done in duplicate for each sample. The bar represents the sample standard deviation of the activity in these samples.

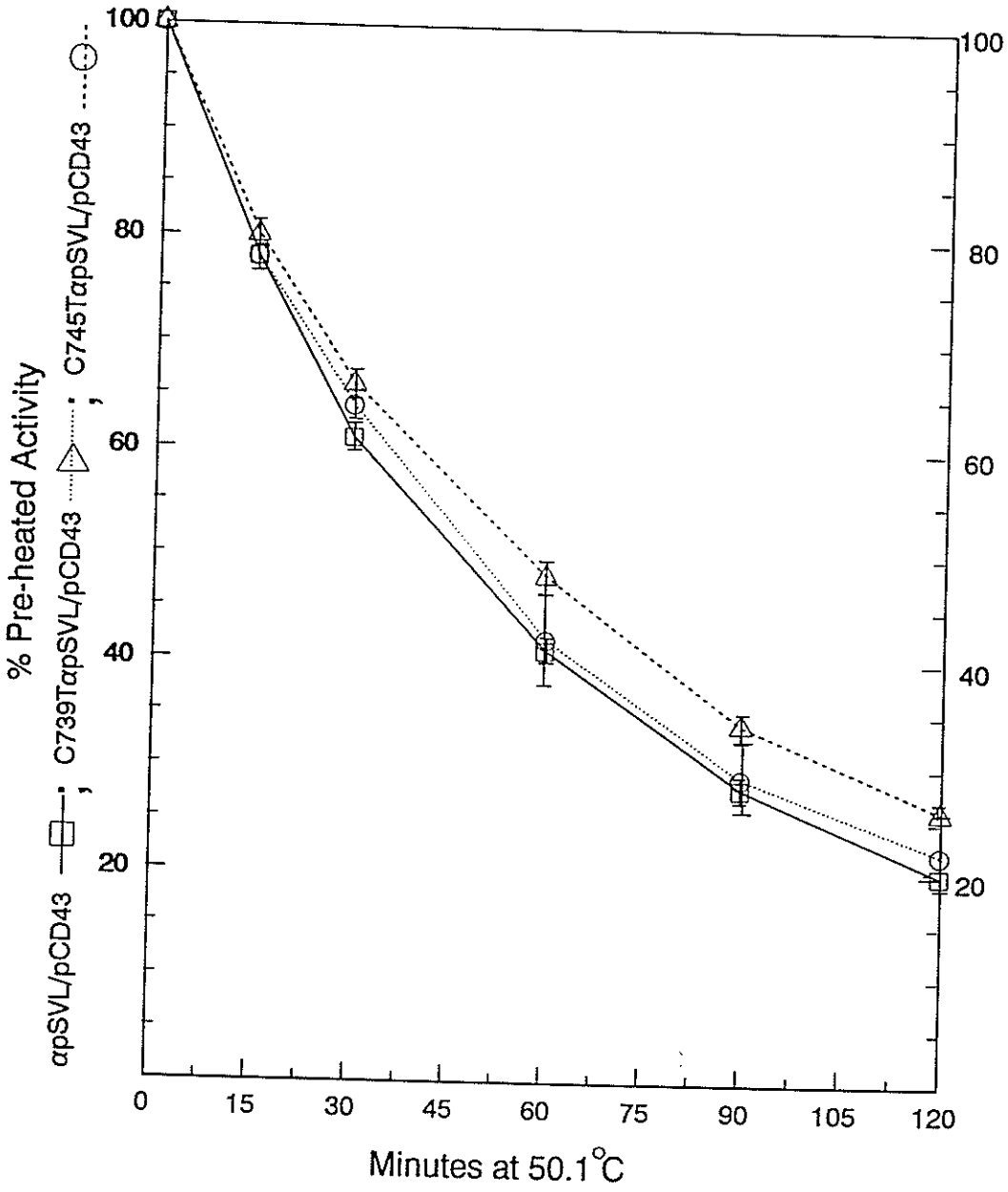

% Pre-heated Activity
αpSVL/pCD43
C739TαpSVL/pCD43
C745TαpSVL/pCD43
100
80
60
40
20
0
15
30
45
60
75
90
105
120
Minutes at 50.1°C

Fig. 19. Heat sensitivity of Hex A from normal and Hex A pseudodeficient fibroblasts. Approximately 125 µg of protein from normal and Hex A pseudodeficient fibroblast cell extracts was incubated at 50.1°C in 200 µl of citrate-phosphate buffer, pH 4.4, in the presence of 0.3% BSA. Aliquots of 10 µl were taken at 0, 15, 30, 60, 90, and 120 minutes of the incubation and used to determine Hex A & S activity using 4-MUGS as the substrate at 37°C. The activities of the 0 minute incubation were defined as 100% and the subsequent values are expressed as a percent of the 0-minute-incubation activity. The data were derived from two experiments with duplicate samples at each point. Assays for Hex A & S activity were done in duplicate for each sample. The bar represents the sample standard deviation of the activity in these samples.

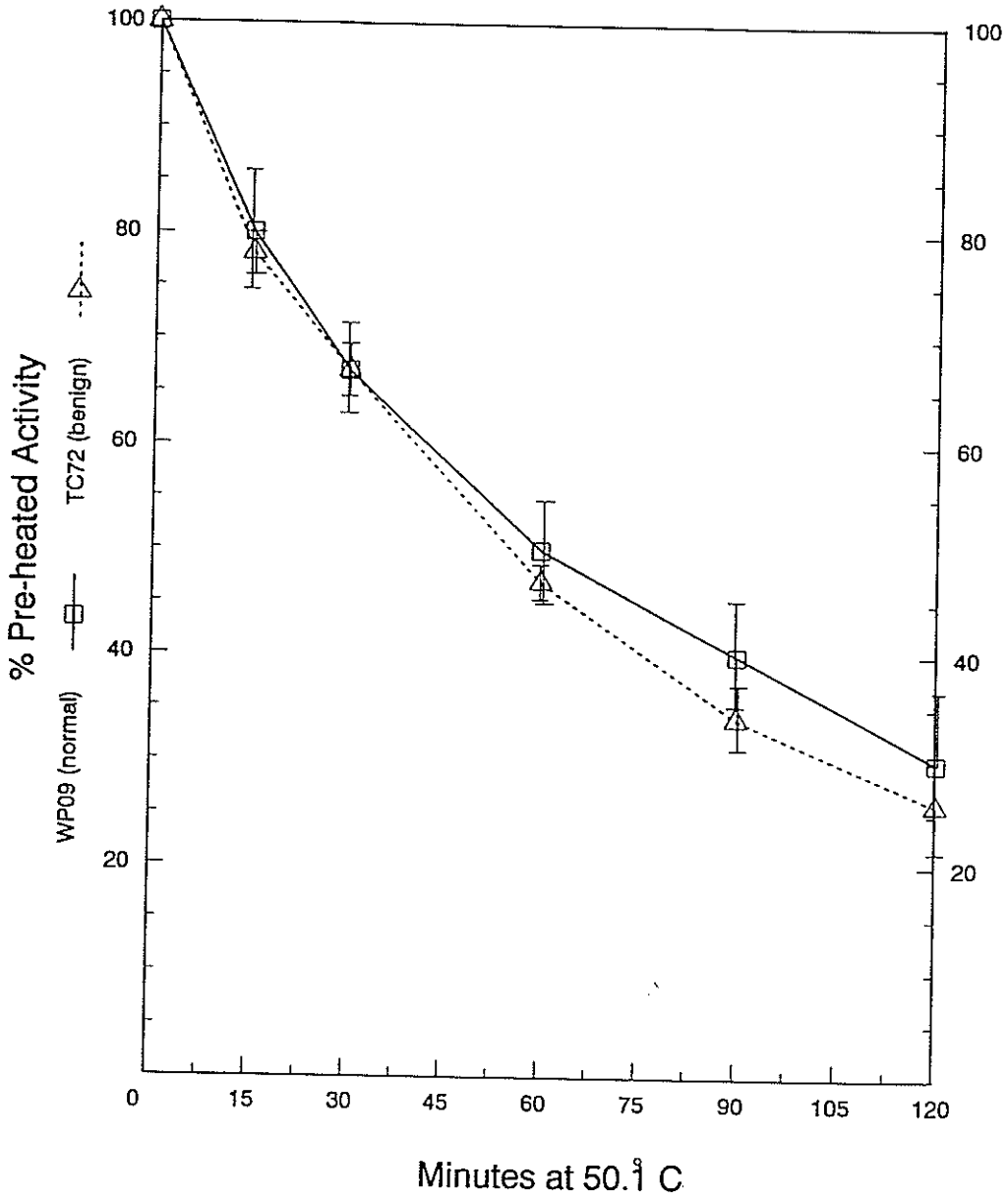

% Pre-heated Activity
WP09 (normal)
TC72 (benign)
100
80
60
40
20
0
15
30
45
60
75
90
105
120
Minutes at 50.1° C

Fig.20. Determination of the pH optimum of Hex A from normal and Hex A pseudodeficient fibroblasts. The Hex A activity in cell extracts from normal and Hex A pseudodeficient fibroblasts was determined in citrate (20 mM)/phosphate (30mM) buffer containing 0.3% BSA, differing in pH and using 4-MUGS as the substrate. The data were derived from two experiments with duplicate samples at each point. Assays for Hex A & S activity were done in duplicate for each sample. The bar represents the sample standard deviation of the activity in these samples.

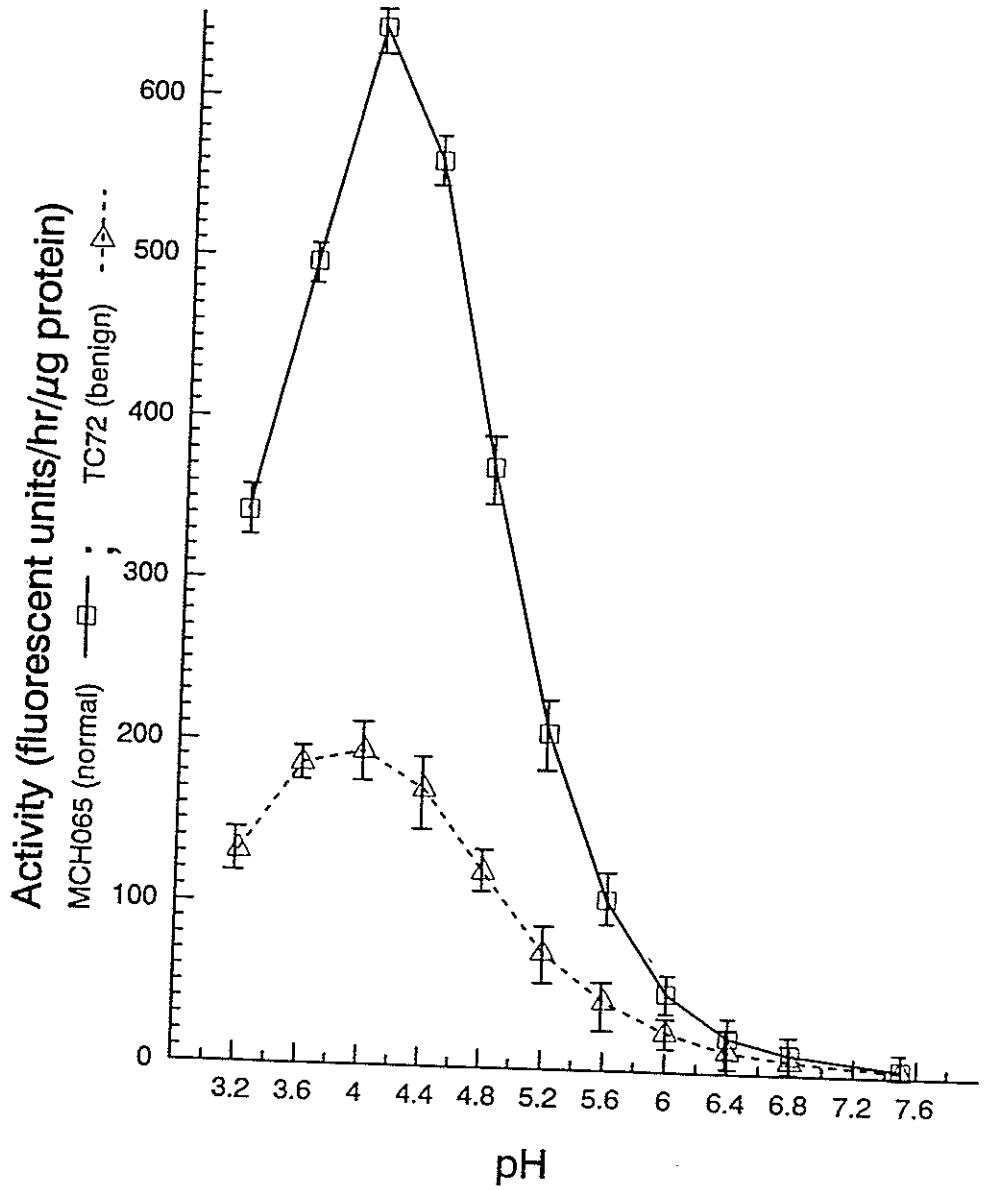
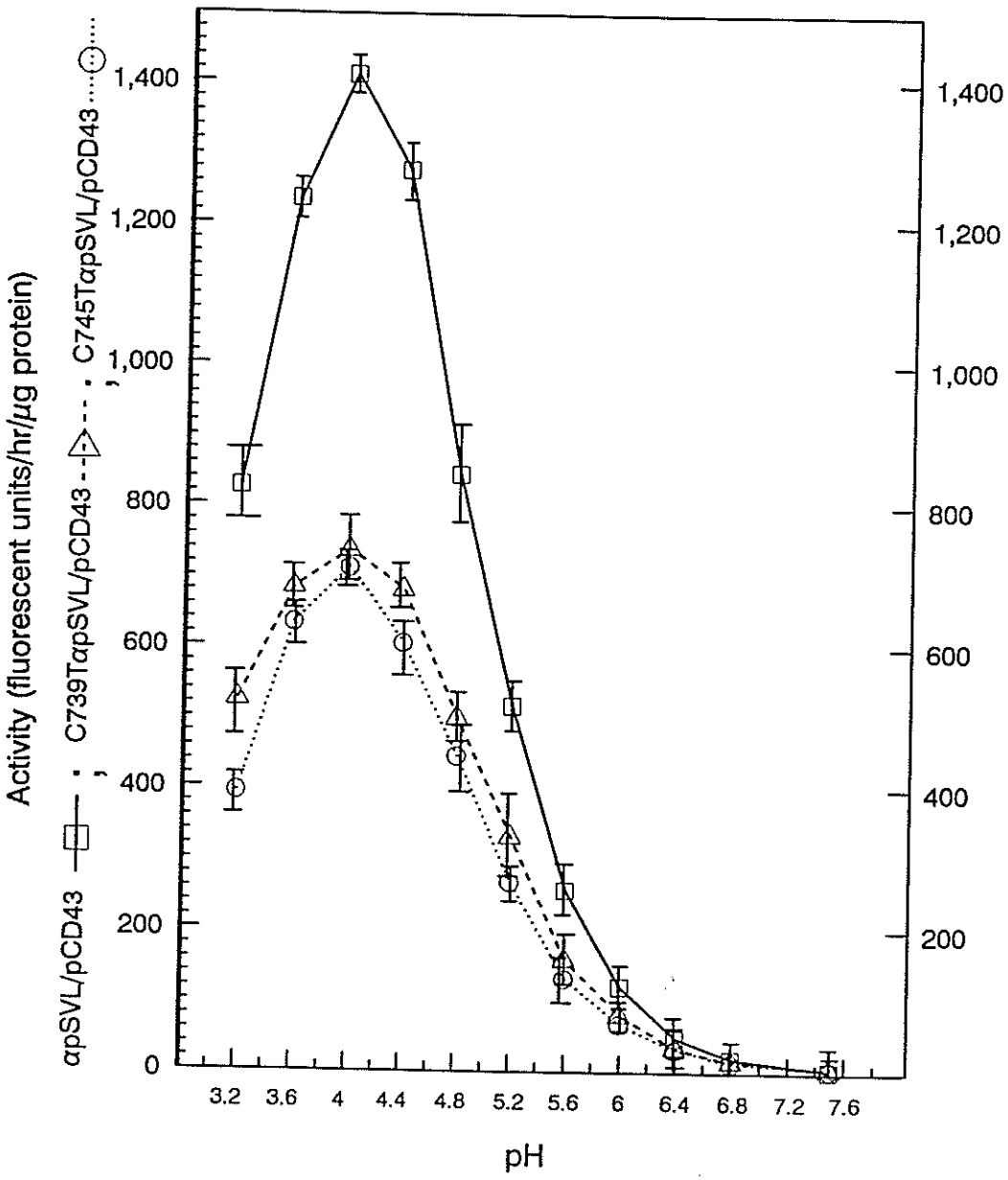

Activity (fluorescent units/hr/μg protein)
MCH065 (normal)
TC72 (benign)
600
500
400
300
200
100
0
3.2
3.6
4
4.4
4.8
5.2
5.6
6
6.4
6.8
7.2
7.6
pH

Fig.21. Determination of pH optimum for the hydrolysis of 4-MUGS by expressed Hex A & S. The Hex A & S activities in cell extracts from pCD43/αpSVL, pCD43/C739TαpSVL, and pCD43/C745TαpSVL transfected Cos-7 cells were determined in citrate (20 mM)/phosphate (30mM) buffer containing 0.6% BSA, differing in pH and using 4-MUGS as the substrate. The data were derived from two experiments with duplicate samples at each point. Assays for Hex A & S activity were done in duplicate for each sample. The bar represents the sample standard deviation of the activity in these samples.


αpSVL/pCD43
C739TαpSVL/pCD43
C745TαpSVL/pCD43
Activity (fluorescent units/hr/μg protein)
0
200
400
600
800
1,000
1,200
1,400
3.2
3.6
4
4.4
4.8
5.2
5.6
6
6.4
6.8
7.2
7.6
pH

normal (WP09) and Hex A pseudodeficient (TC72) fibroblasts, and from Cos-7 cells transfected with pCD43/C745TαpSVL using a DEAE-cellulose column. The fractions corresponding to Hex A were pooled, concentrated, and the apparent $K_m$ and $V_{max}$ toward both 4-MUG and 4-MUGS were determined using direct plot analysis (Henderson, 1993). An example of the use of direct plot analysis to determine the $K_m$ and $V_{max}$ of Hex A from normal fibroblasts towards 4-MUG is shown in Fig.22. A summary of the $K_m$ and $V_{max}$ of normal and mutant Hex A are shown in Table 5. The results revealed that the benign mutations, R247W and R249W, do not have a significant effect on the $K_m$ value of Hex A for the synthetic substrates, 4-MUG and 4-MUGS, although the apparent $V_{max}$ value for both synthetic substrates was substantially decreased by these benign mutations. This indicates that the benign mutations do not affect the affinity of Hex A towards the synthetic substrates. To determine if the lower apparent $V_{max}$ value of Hex A containing the benign mutations was the result of a change in catalytic activity of the α-subunit or a reduction of α-subunit protein in the partially purified Hex A fraction, equal activities toward 4-MUGS were separated by SDS-PAGE and the α-subunit protein levels were analyzed by western blot. Similar levels of α-subunit protein were detected in the normal and Hex A pseudodeficient fibroblasts and the Cos-7 cells transfected with normal and benign-mutation containing αpSVLs (Fig.23). This demonstrated that the benign mutations decreased the apparent $V_{max}$ value of Hex A by reducing the level of α-subunit protein.

3.4 Effect of benign mutations, R247W and R249W, on the processing of the α-subunit of Hex A.

Fig.22. Determination of $K_m$ and $V_{max}$ of Hex A towards 4-MUG using direct linear plot analysis. The Hex A activities (shown on Y-axis) corresponding to the substrate (4-MUG) concentrations, 0.133 mM, 0.33 mM, 0.67 mM, 1.0 mM, 1.33 mM, 2.0 mM, 2.67 mM, 4.07 mM, and 8.133 mM (indicated on X-axis) were determined by incubating an aliquot of the DEAE-column fraction containing partially purified Hex A (0.12 µg protein) in 30 µl of 4-MUG in citrate/phosphate buffer, 0.6% BSA, pH 4.4, at 37°C for 30 min. Nine pairs of velocity (υ or Hex A activity) and substrate concentration (s) were obtained. A υ value was plotted onto the Y or $V_{max}$ axis and the corresponding negative s value was plotted onto the X or $K_m$ axis. A line was drawn through the two points. The intersection points of the lines derived from the seven pairs of υ and s were used to identify the middle value, which was taken as $K_m$ or $V_{max}$. Assays for each pair of υ and s were done in duplicate.

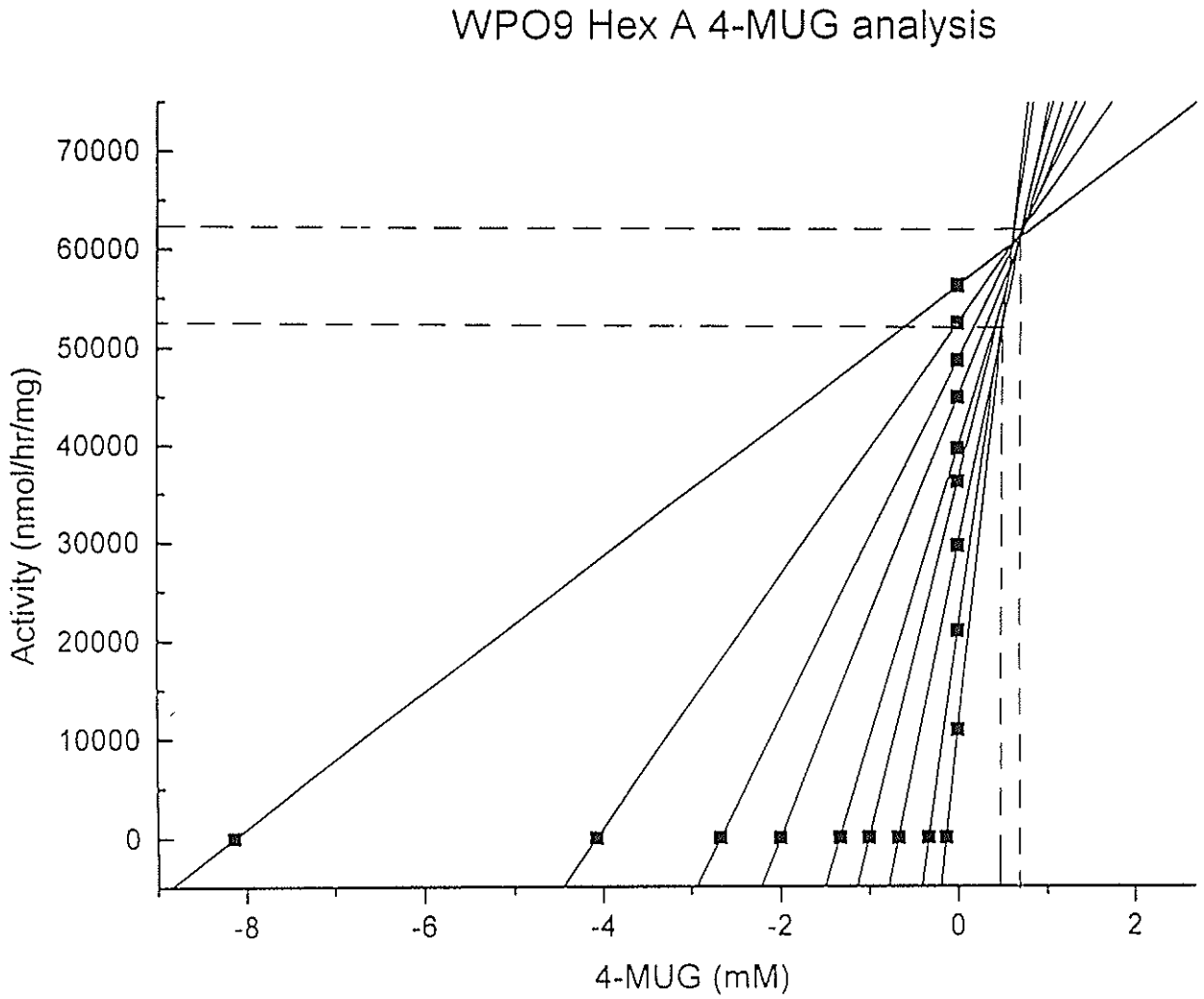

WPO9 Hex A 4-MUG analysis
Activity (nmol/hr/mg)
70000
60000
50000
40000
30000
20000
10000
0
-8
-6
-4
-2
0
2
4-MUG (mM)

Table 5. Kinetic Studies of Hex A[a] Expressed in Cos-7 Cells and Fibroblasts.

| | 4-MUG | | 4-MUGS | |
|---|---|---|---|---|
| | Km (mM) | Vmax($\mu$mol/hr/mg) | Km (mM) | Vmax($\mu$mol/hr/mg) |
| WP09[b] | 0.83 ± 0.17 | 62.0 ± 3.6 | 0.56 ± 0.03 | 2.6 ± 0.22 |
| | n=2 | n=2 | n=2 | n=2 |
| TC72/R247W[b] | 0.65 ± 0.10 | 15.9 ± 1.14 | 0.48 ± 0.11 | 0.60 ± 0.15 |
| | n=2 | n=2 | n=3 | n=3 |
| Cos-7/R249W[c] | 0.60 ± 0.10 | 10.46 ± 0.03 | 0.50 ± 0.10 | 1.07 ± 0.02 |
| | n=2 | n=2 | n=2 | n=2 |

[a] The Hex A activities were determined by incubating a reaction mix, containing a similar level of DEAE-column isolated Hex A activity, with 7 to 9 various concentrations of 4-MUG (0.133 to 8.133 mM) or 4-MUGS (0.133 to 10.66 mM) in citrate/phosphate buffer, 0.3% BSA, pH 4.4, in 30 $\mu$l, at 37°C for 30 min (120 min for 4-MUGS). The apparent $K_M$ and $V_{max}$ values were determined using the direct linear plot method (Henderson, 1993; Fig.22).

[b] Hex A was isolated from normal (WP09) and Hex A pseudodeficient (TC72) fibroblast cells.

[c] Hex A was isolated from pCD43/C745T$\alpha$pSVL-transfected Cos-7 cells.

n-denotes the number of experiments. Each experiment was carried out with duplicate samples. Assays for activities were done in duplicate for each sample.

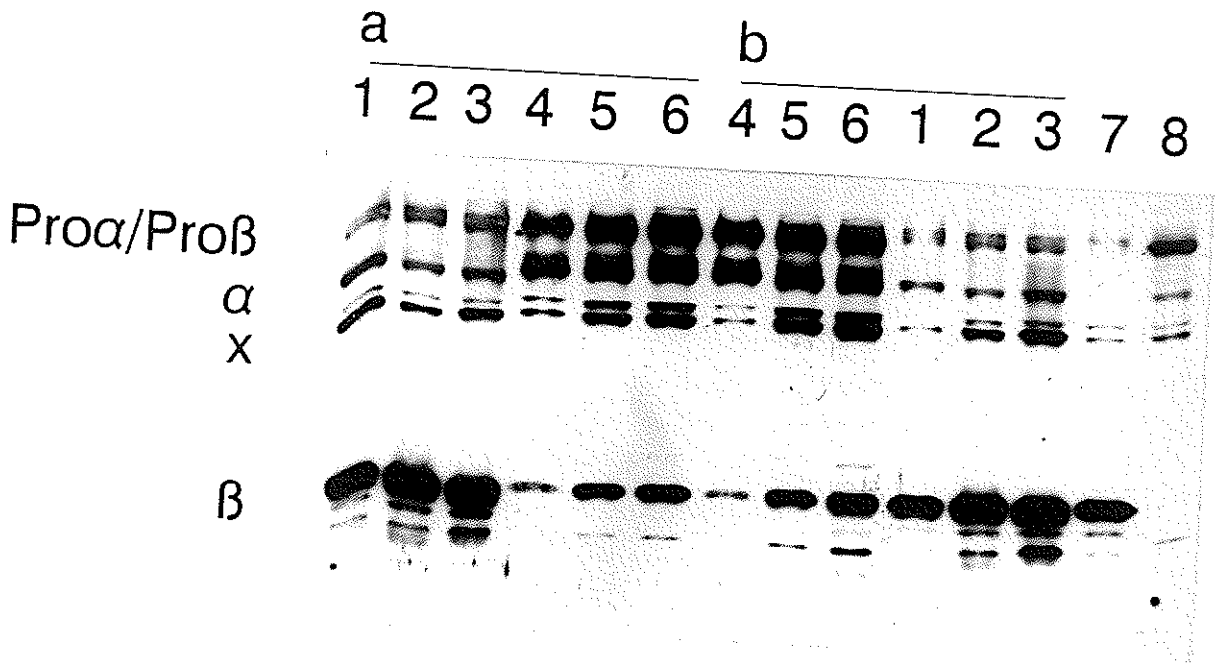



Fig.23. Comparison of the α-subunit protein and Hex A & S activity. Portions of fibroblast cell extracts, containing 8250 fluorescent units/90 min of activity (lanes 1-3), and of transfected Cos-7 cell extracts, containing 4000 fluorescent units/30 min of activity (lanes 4-6), measured using 4-MUGS as the substrate, were separated by SDS-PAGE. Western blot analysis of the protein was done with the polyclonal anti-Hex A antibody. Lanes 1-3: 1, MCH065 (normal); 2, TC72 (benign R247W); 3, GM04863 (benign R247W) and lanes 4-6: 4, pCD43/αpSVL (normal); 5, pCD43/C739TαpSVL (benign); 6, pCD43/C745TαpSVL (benign). Panel a and b indicate cell extracts from duplicate cell culture plates. Lanes 7-8: 7, WG1881 (Tay-Sachs disease); 8, GM00294 (Sandhoff disease).

The results shown in the above sections clearly demonstrated that the benign mutations, R247W and R249W, decreased Hex S and Hex A & S activities by reducing the protein level of the α-subunit. They did not affect the pH optimum for the catalytic reaction towards the synthetic substrates, they did not significantly increase the heat sensitivity *in vitro*, and they did not change the enzyme's affinity to the synthetic substrates. These results suggested that these two benign mutations have similar effects on Hex A. We decided to concentrate our processing studies on a fibroblast cell line containing one benign mutation, R247W, in the subsequent studies.

To determine how the benign mutations reduce the α-subunit protein, we examined the processing of the mutant α-subunit *in vivo*. Hex A in fibroblasts from normal and Hex A pseudodeficient subjects and in transfected Cos-7 cells, was followed from its synthesis and processing to its mature lysosomal form by pulse-labelling with $^{32}P$ or [$^{35}S$] methionine/[$^{35}S$]cysteine and chasing for various intervals. The radio-labelled ß-hexosaminidase was immunoprecipitated, analyzed by high porous gradient SDS-PAGE and detected by fluorography or autoradiography.

Three sources of antibodies with different specificities were used to immunoprecipitate the α-subunit in its different forms. To assess the specificity of these antibodies, the following experiment was initially performed.

3.4.0 Specificity of the antibodies used for immunoprecipitation.

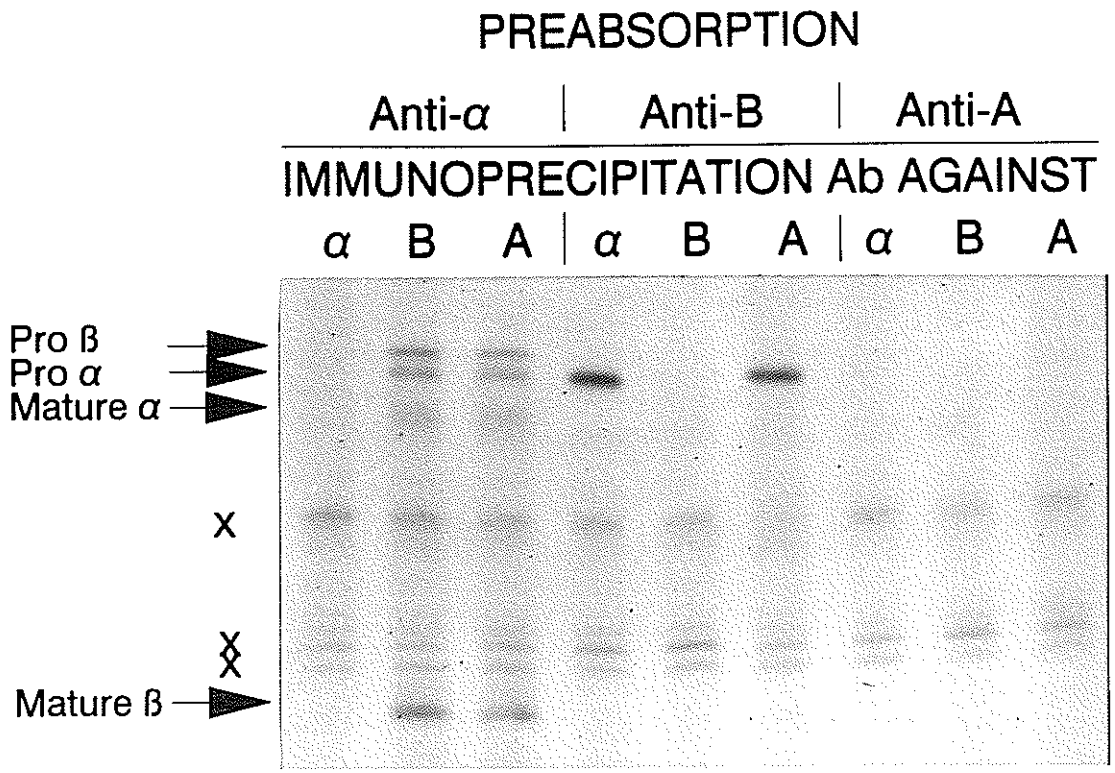
Antibodies against human Hex A, human Hex B and isolated human α-subunit protein were gifts from Dr. R. Gravel (McGill University, Montreal) and Dr. R. Proia (NIH). The

antibodies against Hex B and the isolated α-subunit were previously characterized by Proia *et al.* (1984). To determine the specificity of the Hex A antibody and confirm the specificity of the anti-α-subunit and anti-Hex B antibodies, cell extracts from pulse-labelled normal fibroblasts were preabsorbed with each of the antibodies. The ß-hexosaminidase proteins remaining in the preadsorbed supernatants were immunoprecipitated with each of the antibodies and analyzed by gradient SDS-PAGE. The labelled proteins were visualized by fluorography. The results, as shown in Fig.24, confirmed the observations of a previous study (Proia *et al.*, 1984) that under non-denaturing conditions (see section 2.22.1 Immunoprecipitation using three antibodies), the anti-α-subunit antibody immunoprecipitated the free-form (ie. non-dimerized) precursor α-subunit, but not the ß-subunit-associated α-subunit or the ß-subunit. The anti-Hex B antibody interacted with the precursor and mature ß-subunit and also precipitated the ß-subunit-associated α-subunit, but not the free-form of the α-subunit. The anti-Hex A antibodies precipitated both the free and associated forms of the α-subunit. The results demonstrated the specificity of the anti-Hex A antibody and qualified this antibody for use in the subsequent immunoprecipitation.

### 3.4.1 Effect of the benign mutation, R247W, on the phosphorylation of the α-subunit.

The phosphorylation of the α-subunit is normally a prerequisite for Hex A to be recognized by the mannose-6-phosphate receptor and targeted to the lysosome (see section 1.4). To determine if the benign mutations have an effect on the phosphorylation of the α-subunit, fibroblasts from normal, Hex A pseudodeficient, Tay-Sachs disease and Sandhoff disease subjects were pulse-labelled with $^{32}P$. The radio-labelled forms of ß-hexosaminidase

Fig.24. Determination of specificity of antibodies used for immunoprecipitations. Confluent normal fibroblasts (MCH65) of three (20 x 100 mm) dishes were pulse-labelled with TRAN35S-LABEL (0.3 mCi) for 8 hr. Cell extract for immunoprecipitation was prepared in 1 ml Lysis Buffer containing 1% BSA (see section 2.15.1). For pre-absorption, the cell extract was divided in three (0.33 ml) portions, and each was incubated with 4 µl of antisera against the α-subunit, Hex A, or Hex B at 4°C for 2 hr with agitation. The antibody-antigen complexes were precipitated twice using PANSORBIN as described in section 2.22.1. For immunoprecipitation, supernatants obtained after the preabsorption were diluted to 1 ml with Lysis Buffer containing 1% BSA and again divided into three aliquots. Each aliquot was incubated again with 4 µl of antiserum against the α-subunit, Hex A, or Hex B at 4°C, overnight, with rocking. The antibody/antigen complexes obtained at the second immunoprecipitation were analysed on a high porous 5-12% gradient SDS-PAGE gel and the radiolabelled proteins were detected by fluorography.


PREABSORPTION
Anti-α
Anti-B
Anti-A
IMMUNOPRECIPITATION Ab AGAINST
α B A α B A α B A
Pro ß
Pro α
Mature α
X
X
X
Mature ß

were immunoprecipitated using anti-α-subunit, anti-Hex B and anti-Hex A antibodies, separated by gradient SDS-PAGE and detected by autoradiography. In three separate experiments, a considerable amount of precursor α-subunit was observed in the free- and ß-subunit-associated form in the sample from benign fibroblasts (Fig.25). The same pattern was observed in the samples from normal fibroblasts suggesting that Hex A containing the benign mutation R247W was normally phosphorylated. As expected, Tay-Sachs disease samples did not show a $^{32}$P-labelled band corresponding to the α-subunit and the Sandhoff disease samples did not exhibit a band corresponding to the $^{32}$P-labelled ß-subunit.

The normal phosphorylation of Hex A containing the benign mutation indicated that the benign mutation did not affect the α-subunit folding or glycosylation which would both be necessary for phosphorylation.

3.4.2 Effect of the benign mutation R247W on the secretion of Hex A.

Normally, most Hex A is targeted to the lysosome, although some Hex A molecules are secreted extracellularly after α- and ß-subunit dimerization (reviewed by Gravel *et al.*, 1995). To assess the effects of the benign mutations on the extracellular secretion of Hex A, fibroblasts from a Hex A pseudodeficient subject were labelled with $^{35}$S-labelled methionine and cysteine for 3 hr. This was chased for 18 hr in the presence of α-MEM supplemented with 10 mM $NH_4Cl$. Fibroblasts from the normal control were treated identically. Medium extracts were prepared and the radio-labelled molecules secreted into the medium were immunoprecipitated and analyzed.

The results in Fig.26 showed that the secretion of the precursor α-subunit from Hex

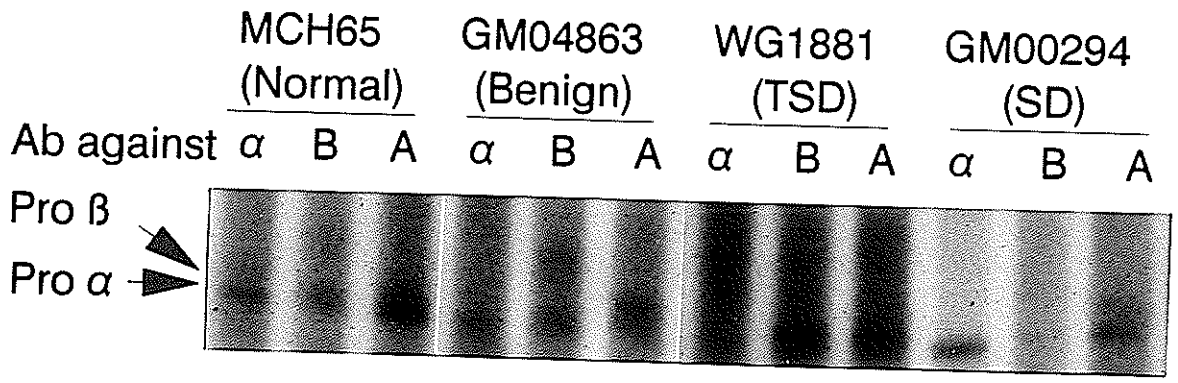



Fig.25. Effect of the C739T(R247W) on the phosphorylation of the α-subunit of Hex A. Fibroblasts from four cell lines, as indicated, were grown to confluence in cell culture dishes (20 x 100 mm). Each cell line was pulse-labelled with $^{32}P$ (0.3 mCi) for 3 hr and cell extracts were prepared in Lysis Buffer containing 1% BSA (see section 2.15.1). The anti-α-subunit, anti-Hex A and anti-Hex B antibodies were used for immunoprecipitation and one third of the sample was analysed on a high porous 5-12% gradient SDS-PAGE gel. The radioactive signals were detected using Kodak X-OMAT-AR film. This autoradiogram represents a one week exposure.

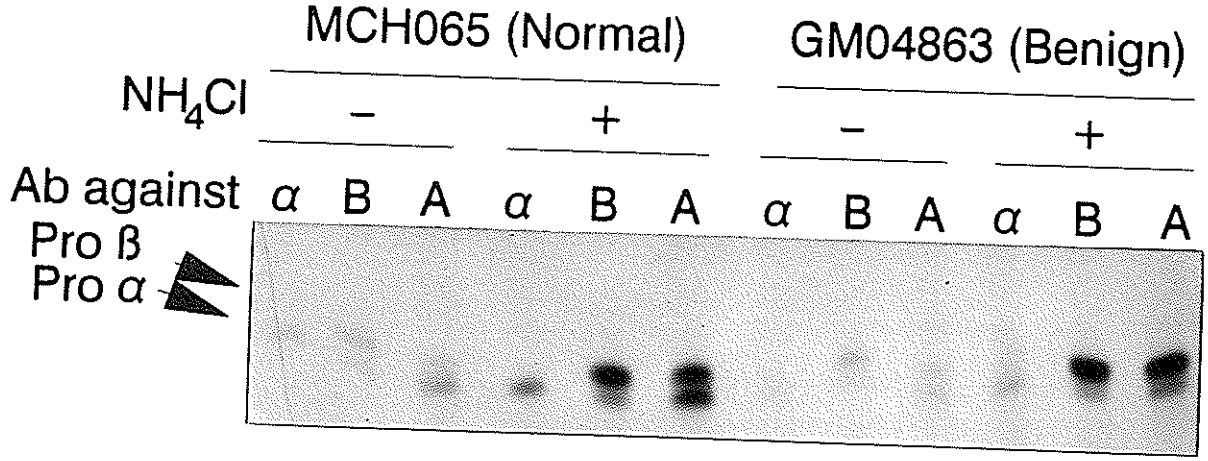



Fig.26. Effect of the C739T(R247W) on the secretion of Hex A. Fibroblasts from two cell lines, as indicated, were grown to confluence in tissue culture dishes (20 x 100 mm). Each cell line was pulse-labelled with TRAN35S-LABEL (0.3 mCi) for 3 hr and chased for 20 hr in the presence (+) and absence (-) of 10 mM $NH_4Cl$. The medium extracts were prepared (see section 2.15.1) and similar amounts of protein were analysed. Three antibodies, as indicated, were used for immunoprecipitation and one fourth of the sample was analysed on a high porous 5-12% gradient SDS-PAGE gel. The radioactive signals were detected by fluorography. Fetal bovine serum was not used in the culture medium to avoid the hydrolysis of the secreted α-subunit by the proteases which may be present in the fetal bovine serum.

A pseudodeficient fibroblasts was enhanced by growth in the presence of $NH_4Cl$. The α-subunit in free form, immunoprecipitated by anti α-subunit antibody, was almost undetectable and a very low level of the α-subunit was seen in ß-subunit associated form without the presence of $NH_4Cl$ in the medium. However, when the cells were incubated in the presence of $NH_4Cl$, the α-subunit in all forms became obvious. The same results were also observed in the normal fibroblasts (Fig.26). This experiment was repeated three times and similar results were obtained. This indicates that the benign mutations do not affect the normal secretion of Hex A. This also indirectly suggests that benign mutations do not interfere with the processing and targeting of Hex A; the effect of $NH_4Cl$ on the dissociation of Hex A and the mannose-6-phosphate receptor occurs in the pre-lysosomal and lysosomal compartments (Seglen, 1983). The increased level of the ß-subunit precursor in the presence of $NH_4Cl$ was also observed in all fibroblasts.

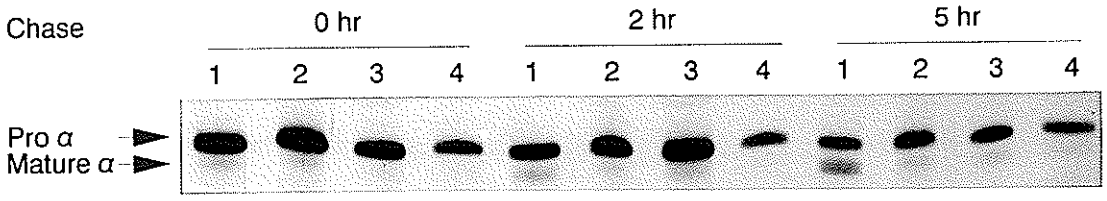
The results obtained from these studies, and those of the previous sections, suggested that the benign mutation R247W did not affect the phosphorylation, secretion or targeting of the α-subunit *in vivo*. The studies in the following sections were done to determine if the benign mutations affect the stability of the α-subunit *in vivo*. Both the transiently expressed Hex S in Cos-7 cells and Hex A in the fibroblasts from normal and Hex A pseudodeficient subjects were used. The transient expression system was used to provide the Hex S containing the benign mutation R249W because fibroblasts containing this mutation were not available. This also decreased the interference from the intermediate un-processed ß-subunit that might exist in the pseudodeficient fibroblasts.

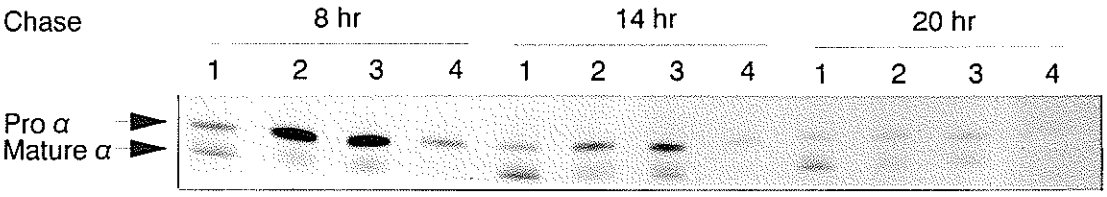
3.4.3 Effect of benign mutations on the stability of the α-subunit expressed as Hex S in Cos-7 cells.

To determine the effect of the benign mutations on α-subunit stability, C739TαpSVL and C745TαpSVL were transfected into Cos-7 cells to express mutant Hex S. The normal α-subunit expression vector, αpSVL, and the adult-onset disease mutation-containing vector, G805AαpSVL, were also transfected as controls. ß-Galactosidase was co-expressed to determine the transfection efficiency. The transfected Cos-7 cells were pulsed for 3 hr and chased for various intervals from 0 hr to 20 hr. Equal amounts of normalized cell extract protein, based on the level of expressed ß-galactosidase activity from each sample, were used for immunoprecipitation with anti-Hex A antibody and analyzed by gradient SDS-PAGE. The radioactive proteins were detected by fluorography.

The results showed that after a 3 hr pulse, the precursor α-subunit was produced from both the normal and mutant αpSVLs which included vectors carrying two benign mutations, C739T and C745T, and an adult-onset mutation, G805A (Fig.27). The benign mutation-containing precursor α-subunits were present throughout the chase time and appeared to have similar to or higher levels than the normal. The G805A mutation-containing precursor α-subunit had disappeared by 20 hr of chase. The level of the mature α-subunit from the wild-type αpSVL reached a maximum after 5 hr of chase and remained at the same level up-to 14 hr, then slightly decreased at 20 hr of chase. Levels of both benign mutation-containing mature α-subunits reached a maximum at about 8 hr of chase, and dramatically decreased by 20 hr of chase. The mature α-subunit from G805AαpSVL was also detectable up to 8 hr of chase. The results also showed a higher ratio of the precursor to the mature form of the

Fig.27. Effect of benign mutations on the stability of the α-subunit as Hex S expressed in Cos-7 cells. Cos-7 cells were transfected with normal and mutant α-subunit cDNA to make Hex S and together with pRc/CMV-ß-gal to express ß-galactosidase as a measure of transfection efficiency. The transfected cells (12 x $10^6$) from four separate electroporations were pooled, mixed and evenly dispensed into six tissue culture dishes (20 x 100 mm). The medium was changed at 24 hr post-transfection and the cells were pulse-labelled for 3 hr with TRAN35S-LABEL (0.15 mCi) at 45 hr post-transfection. These were chased for various intervals as indicated. Cells were harvested in 0.5 ml Lysis Buffer and stored at -80°C. Cell extracts were prepared for immunoprecipitation after thawing. Two µl of cell extract was used for the protein assay and 3 µl of cell extract was used to determine ß-galactosidase activity levels. Normalized samples (~0.7 mg protein), based on the protein concentration and normalization factor derived from ß-galactosidase activity, was taken from each sample and the volume was equalized to 0.8 ml with Lysis Buffer containing 1% BSA. Antiserum against Hex A was used for immunoprecipitaion (see section 2.22.1). One quarter of the sample was analysed on a high porous 5-12% gradient SDS-PAGE gel and the labelled proteins were detected by flurography. Lane 1, αpSVL; lane 2, C739TαpSVL; lane 3, C745TαpSVL; lane 4, G805AαpSVL.


Chase
0 hr
2 hr
5 hr
1 2 3 4 1 2 3 4 1 2 3 4
Pro α
Mature α


Chase
8 hr
14 hr
20 hr
1 2 3 4 1 2 3 4 1 2 3 4
Pro α
Mature α

benign mutation-containing α-subunit than that of the normal. These results suggested that the mature α-subunits containing benign mutations were not as stable as the normal α-subunit, but more stable than the α-subunit containing the adult-onset G805A mutation. Further, the benign mutations appeared to decrease the rate of conversion from the precursor α-subunit to its mature form.

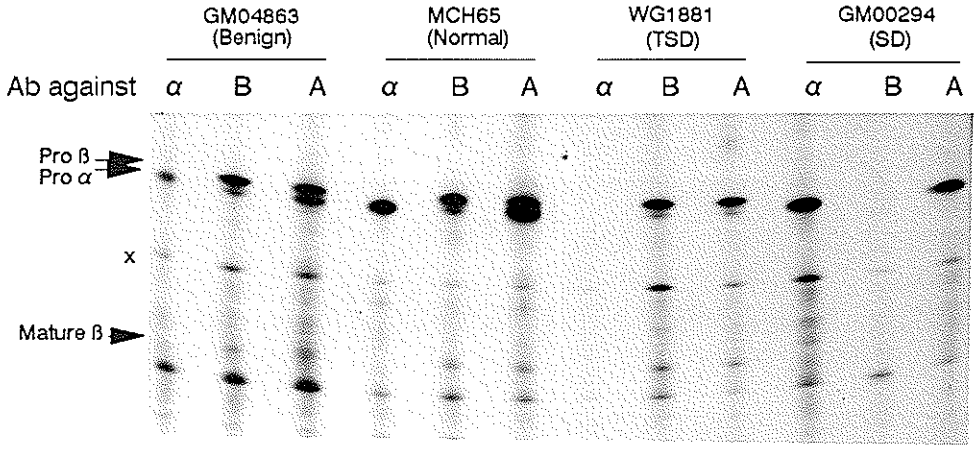
### 3.4.4 Effect of the benign mutation R247W on the stability of the α-subunit in Hex A pseudodeficient fibroblasts.

To determine the effect of the benign mutation R247W on the stability of the α-subunit of Hex A *in vivo,* fibroblasts from a Hex A pseudodeficient subject were pulse-labelled for 3 hr and chased for different periods of time. Fibroblasts from a normal individual, Tay-Sachs disease and Sandhoff disease patients were treated identically. The radio-labelled ß-hexosaminidase in cell extracts, equalized on the basis protein, were immunoprecipitated and analyzed by gradient SDS-PAGE, then detected by fluorography. Two methods for immunoprecipitation were used. In the first method, three types of antibodies, anti-Hex A, anti-Hex B and anti-α-subunit antibodies, were used to separately immunoprecipitate free-form α-subunit, ß-subunit-associated α-subunit and total α-subunit (see section 2.22.1). In the second method, the total α-subunit was immunoprecipitated with anti-Hex A antibody and denatured to release the associated form of the α-subunit. The anti-α-subunit antibody was then used to immunoprecipitate the total α-subunit which was now monomeric (see section 2.22.1 Immunoprecipitation using anti-α-subunit antibody).

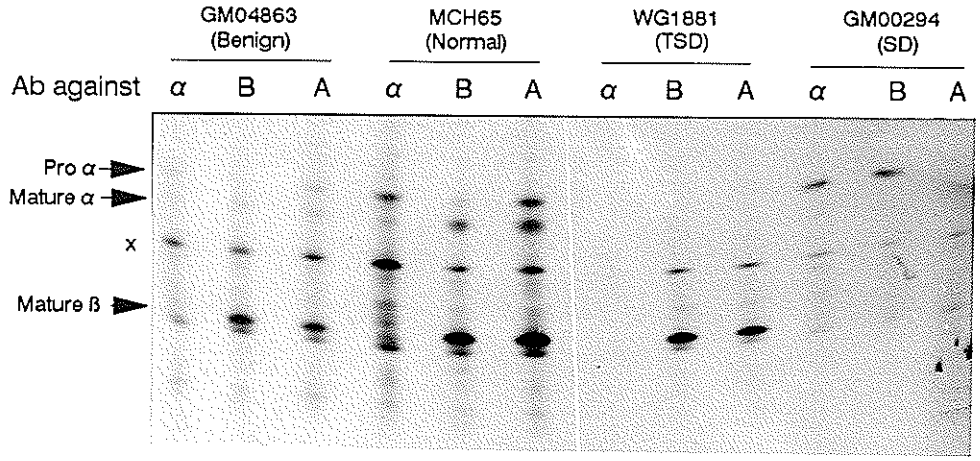
Using the first method, the results (Fig.28) showed that after 3 hr of labelling, the

Fig.28. Effect of benign mutation R247W on the stability of the α-subunit of Hex A in fibroblasts--immunoprecipitation with three antibodies. Fibroblasts from four cell lines, as indicated, were grown to confluence in tissue culture dishes (20 x 100 mm). Each cell line was pulse-labelled with TRAN35S-LABEL (0.3 mCi) for 3 hr and chased for various intervals as indicated. The cell extracts were prepared in 0.5 ml Lysis Buffer for immunoprecipitation. Two µl of cell extract was used for a measurement of the protein concentration and 380-450 µl of cell extract, containing an equal amount of protein (1.4 mg), was mixed with Lysis Buffer containing 1% BSA to a final volume of 1 ml. The cell extracts were precleared with anti-fibronectin antiserum and PANSORBIN (see section 2.22.1), and equally divided into three aliquots. Each aliquot (0.33 ml) was used for immunoprecipitation with one of the three antibodies indicated. One fourth of the sample was analysed on a high porous 5-12% gradient SDS-PAGE gel. The labelled proteins were detected by fluorography.

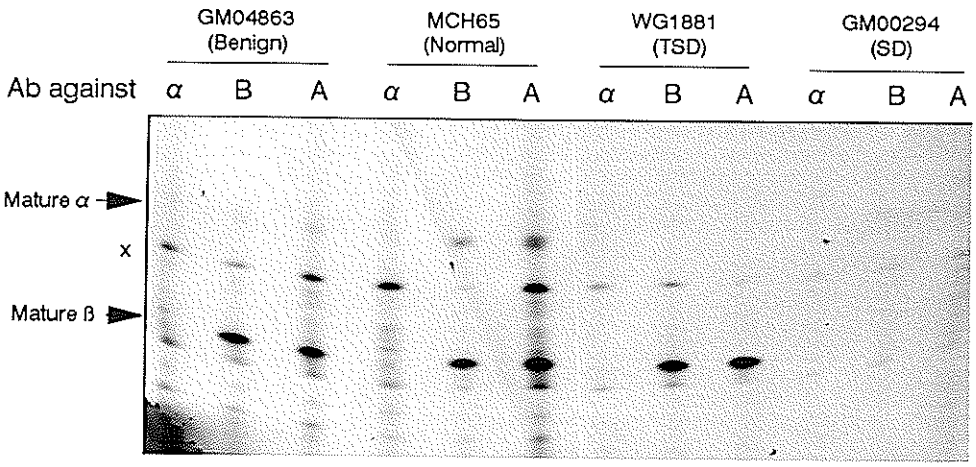
Chase 0 hr




Chase 8 hr




Chase 17 hr



precursor α-subunit protein in the Hex A pseudodeficient fibroblasts appeared lower than that in the normal. This can partially be accounted for by the fact that Hex A pseudodeficient fibroblasts contained a Tay-Sachs disease mutation on the second allele that did not produce α-subunit protein. At 8 hr and 17 hr of chase, the normal fibroblasts showed higher levels of mature α-subunit than the benign mutation-containing fibroblasts. As expected, the Tay-Sachs disease fibroblasts had no α-subunit and the Sandhoff disease fibroblasts had no ß-subunit.

A band that migrated in a similar position to the precursor α-subunit, the bands in lanes B and A at chase 0 hr at the similar level to the Pro-α (Fig.28), had to be considered in the interpretation of the levels of the precursor α-subunit. The identity of this band was based on its presence in the Tay-Sachs disease fibroblasts, but not in the Sandhoff disease fibroblasts (Fig.28, Chase 0 hr). This suggested that this band was related to the ß-subunit and that it might also exist in the normal and pseudodeficient fibroblasts. This band must be the intermediate form of the ß-subunit, $I_1$, demonstrated by Mahuran *et al.* (1985) with a molecular mass of 58 kDa. The presence of this band interfered with the estimation of the level of the free- and the associated-forms of the α-subunit precursor.

The second method used for immunoprecipitation was based on the finding by Hasilik and Neufeld (1980b) that the anti-α-subunit antibody, in the presence of SDS, precipitated both the precursor and mature forms of the α-subunit. A method was developed (see section 2.22.1 Immunoprecipitation using anti-α-subunit antibody) that included a primary immunoprecipitation with anti-Hex A antibody to precipitate the total α-subunit, followed by a denaturation step to dissociate the ß-subunit-associated α-subunit, and finally a secondary

immunoprecipitation with the anti-α-subunit antibody to precipitate the total α-subunit. Using this method, we avoided immunoprecipitating the ß-subunit, and the complications of interpreting the results in the presence of ß-subunit forms that migrated in the same position as the α-subunit.

The fibroblasts from normal, Hex A pseudodeficient, Tay-Sachs disease and Sandhoff disease subjects were grown to confluence, pulse-labelled with $^{35}$S-labelled methionine and cysteine for 3 hr and then chased for different periods of time. An equal amount of protein from the cell extracts prepared from normal and benign mutation-containing fibroblasts were used for immunoprecipitation; the protein levels of cell extracts from the control disease fibroblasts were not normalized. The immunoprecipitated radioactive-labelled α-subunit was separated on the gradient SDS-PAGE gel and detected by fluorography. The results in Fig.29 showed that the benign mutation-containing fibroblasts, at 3 hr of pulse, produced about half the level of the precursor α-subunit when compared to the normal fibroblasts. The normal mature α-subunit level reached near maximum after 5 hr of chase and stayed at a similar level even at 20 hr of chase. The benign mutation-containing α-subunit reached to its maximum level at about 8 hr of chase, stayed at a similar level until 14 hr of chase and decreased at 20 hr of chase. The Tay-Sachs disease fibroblasts had no α-subunit and the Sandhoff disease fibroblasts showed the α-subunit. Similar results were obtained from two separate experiments.

3.5 Effect of E-64 and Leupeptin on the stability of the α-subunit with the benign R247W mutation.

Fig.29. Effect of the benign mutation R247W on the stability of the α-subunit of Hex A in fibroblasts–immunoprecipitation with antiserum against the α-subunit. Fibroblasts from four cell lines, as indicated, were grown to confluence in tissue culture dishes (20 x 100 mm). Each cell line was pulse-labelled with TRAN35S-LABEL (0.3 mCi) for 3 hr and chased for various intervals as indicated. The cell extracts were prepared for immunoprecipitation in 0.65 ml Lysis Buffer and 2 µl of cell extract was used for the measurement of the protein concentration. Cell extracts (480-510 µl) containing equal amounts of protein (1.20 mg) were mixed with Lysis Buffer containing 1% BSA to a final volume of 600 µl. Primary and secondary immunoprecipitations were then done as described in section 2.22.1 (Immunoprecipitation Using Anti-α-subunit Antibody). One fourth of the sample was analysed on a high porous 5-12% gradient SDS-PAGE gel. The labelled proteins were detected by fluorography.

Pro α
Mature α
Chase
0 hr
MCH65 (Normal)
GM04863 (Benign)
WG1881 (TSD)
GM00294 (SD)
2 hr
MCH65 (Normal)
GM04863 (Benign)
5 hr
MCH65 (Normal)
GM04863 (Benign)
8 hr
MCH65 (Normal)
GM04863 (Benign)
14 hr
MCH65 (Normal)
GM04863 (Benign)
20 hr
MCH65 (Normal)
GM04863 (Benign)
WG1881 (TSD)
GM00294 (SD)

To determine if the lysosomal protease inhibitors, E-64 and Leupeptin, could stabilize or elevate the level of the mature α-subunit harbouring the benign R247W mutation, fibroblasts from normal and Hex A pseudodeficient subjects were pulse-labelled with [$^{35}$S]methionine/cysteine for 3 hr and chased for 20 hr in the presence of 280 μM E-64 or 105 μM Leupeptin. The Leupeptin concentration 105 μM was chosen according to von Figura *et al.* (1983). In the experiments with Leupeptin, Tay-Sachs disease and Sandhoff disease fibroblasts were identically treated, but in the absence of Leupeptin in the tissue culture medium. Equal amounts of normal and pseudodeficient cell extract protein were separated by gradient SDS-PAGE and the radioactive proteins were detected by fluorography.

The normal and benign mutation-containing fibroblasts, which were grown in the presence of E-64, showed a partial blockage of the conversion from the α-subunit precursor to the mature form (Fig.30). This indicated an inhibitory effect of E-64 on the lysosomal proteolytic process. No increase in the level of the mature α-subunit was observed in the normal or benign mutation-containing fibroblasts. Bestatin (Boehringer Mannheim Co. Indianapolis, IN), a mammalian cell surface aminopeptidase inhibitor, was used as a control and did not exhibit any effect on the protein level of the α-subunit.

The fibroblasts from normal and Hex A pseudodeficient subjects showed no increase in the α-subunit precursor or mature forms after treatment with Leupeptin (Fig.31). Once again, as expected, the Tay-Sachs disease fibroblasts exhibited no α-subunit and the Sandhoff disease fibroblasts exhibited no ß-subunit. The data are representative of two repeated experiments. Leupeptin has successfully been used to increase the activity and protein levels of arylsulfatase A in the fibroblasts from adult metachromatic leukodystrophy (von Figura *et al.*, 1983).

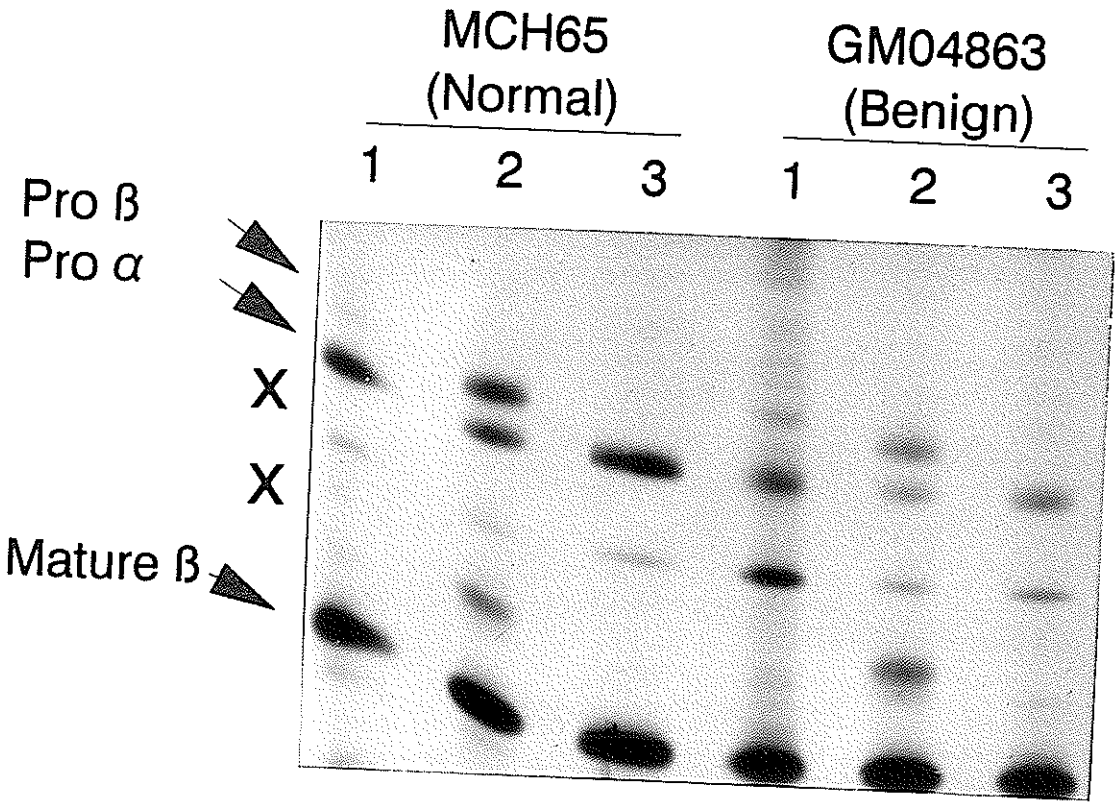
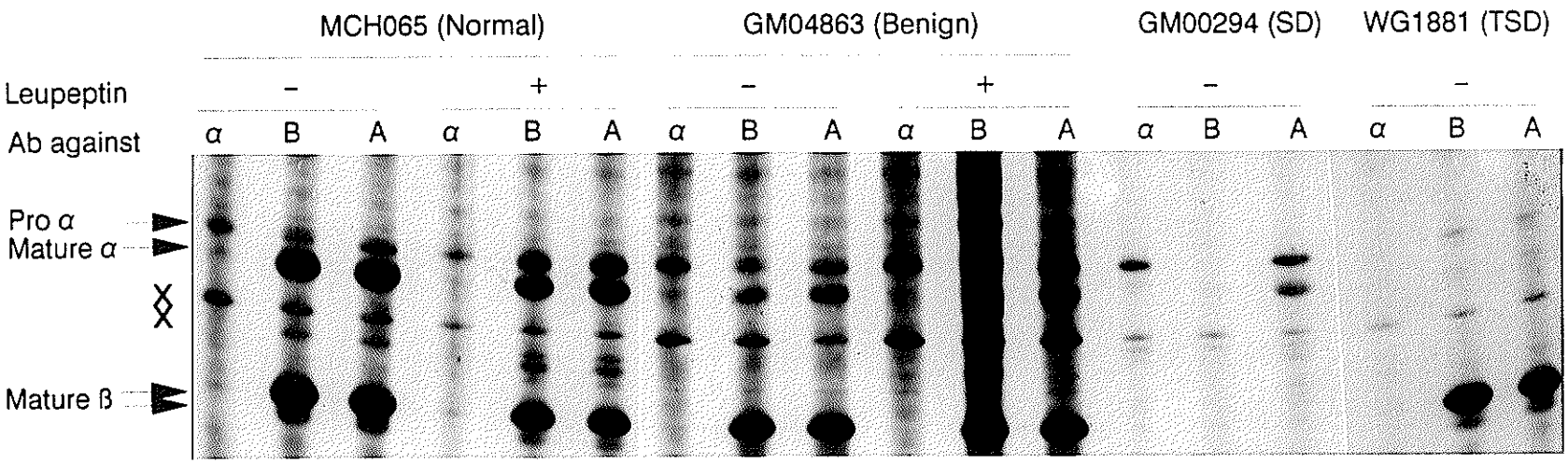



Fig.30. Effect of E-64 on the stability of the α-subunit with the R247W benign mutation. Fibroblasts from two cell lines, as indicated, were grown to confluence in tissue culture dishes (20 x 100 mm). Each cell line was pulse-labelled with TRAN35S-LABEL (0.3 mCi) for 3 hr and chased for 20 hr in the presence of 280 μM E-64 or 87 mM Bestatin. The cell extracts were prepared in 0.5 ml Lysis Buffer for immunoprecipitation. Cell extract protein (1.10 mg) was mixed with Lysis Buffer containing 1% BSA to a final volume of 600 μl. This was used for immunoprecipitation with antiserum against Hex A. One fourth of the sample was analysed on a high porous 5-12% gradient SDS-PAGE gel. The labelled proteins were detected by fluorography. Lane 1, no inhibitors; lane 2, E-64; lane 3, Bestatin.

Fig. 31. Effect of Leupeptin on the stability of the α-subunit with the R247W benign mutation. Fibroblasts from four cell lines, as indicated, were grown to confluence in tissue culture dishes (20 x 100 mm). Each cell line was pulse-labelled with TRAN35S-LABEL (0.3 mCi) for 3 hr and chased for 12 hr in the presence (+) and absence (-) of 105 μM leupeptin. The cell extracts were prepared in 0.5 ml Lysis Buffer for immunoprecipitation. Cell extract protein (1.8 mg) was mixed with Lysis Buffer containing 1% BSA to a final volume of 600 μl. This was used for immunoprecipitation with the antibodies indicated. One fourth of the sample was analysed on a high porous 5-12% gradient SDS-PAGE gel. The labelled proteins were detected by fluorography.


MCH065 (Normal)
GM04863 (Benign)
GM00294 (SD)
WG1881 (TSD)
Leupeptin
–
+
–
+
–
–
Ab against
α B A α B A α B A α B A α B A α B A
Pro α
Mature α
Mature ß

142 a

# 4. DISCUSSION

In humans, ß-hexosaminidase A is the only lysosomal enzyme capable of *in vivo* $G_{M2}$ ganglioside hydrolysis. Deficiency of this enzyme results in Tay-Sachs disease, an autosomal-recessively inherited lysosomal storage disorder with mental and physical manifestations. Although currently incurable, Tay-Sachs disease prevention programs have successfully reduced the disease incidence in the past more than two decades through enzyme screening (Kaback *et al.*, 1993). During routine screening for Tay-Sachs disease carriers and in subsequent prenatal diagnoses, Hex A pseudodeficient subjects were found (Vidgoff *et al.*, 1973; Kelly *et al.*, 1976; O'Brien *et al.*, 1978; Thomas *et al.*, 1982; Grebner *et al.*, 1986; Navon *et al.*, 1986; Triggs-Raine, 1992). Benign mutations, C739T(R247W) and C745T(R249W), associated with Hex A pseudodeficiency in these subjects were also identified (Triggs-Raine *et al.*, 1992; Cao *et al.*, 1993). The ability to identify benign mutations using DNA-based procedures was very important because the existing biochemical screening methods cannot differentiate individuals harbouring benign mutations from those with disease-causing mutations. Further, the existing methods did not allow patients to be readily and reliably differentiated from Hex A pseudodeficient subjects. In the past, these difficulties have resulted in misleading counselling and incorrect prenatal diagnoses.

The specific goals of these studies were to determine: 1. the frequency of the benign mutation C745T(R249W) among the enzyme-defined carriers; 2. if the benign mutations, C739T(R247W) and C745T(R249W), result in Hex A deficiency; 3. if the benign mutations could be differentiated from the disease-causing mutations by expression analysis; 4. the effects of these benign mutations on ß-hexosaminidase A.

A diagnostic strategy to specifically detect the benign C745T(R249W) mutation was

developed. Using this strategy, the pseudodeficient proband, 16819, was confirmed to have the C745T base change inherited from her father (Fig.2). This made her a compound heterozygote for the C745T(R249W) benign mutation and the G805A(G269S) mutation, associated with the adult-onset form of $G_{M2}$ gangliosidosis. This was the first case where a benign mutation had been identified in compound heterozygosity with a mutation that was not associated with classical infantile Tay-Sachs disease. Although this subject was healthy at 42 years of age, the presence of the adult-onset allele might delay the onset of neurological symptoms in this subject. Therefore the possibility that symptoms could develop in this individual cannot be ruled out. However, individuals homozygous for the G805A(G269S) adult-onset mutation have always developed symptoms before 40 years of age (Navon *et al.*, 1990). This, together with the fact that the C745T(R249R) mutation has not been indentified in individuals with any forms of $G_{M2}$ gangliosidosis, suggests that it is a benign mutation.

To analyze the impact of the benign mutation, C745T(R249W), on enzyme-based population screening, the carrier frequency for this mutation in the Jewish and non-Jewish population was estimated. As expected, this mutation was found only among enzyme-defined carriers and not in obligate carriers or non-carriers (Table 1). This provided evidence that this mutation was associated with enzyme deficiency, but may not be associated with disease. This mutation accounted for about 6% (4/63) of enzyme-defined carriers in the non-Jewish population, but it was not detected in Ashkenazi Jewish carriers (0/218). In combination with the frequency of the first benign mutation, C739T(R247W), these two benign mutations accounted for approximately 38% of non-Jewish enzyme-defined carriers (Triggs-Raine *et al.*, 1992; Cao *et al.*, 1993; Kaback *et al.*, 1993; Tomczak et al., 1993). Ours and other

studies indicated that about 38% of non-Jewish and approximately 2% of Ashkenazi Jewish enzyme-defined carriers had a benign mutation and were not at-risk for Tay-Sachs disease.

The impact of the benign mutations also extended to Tay-Sachs disease prevention programs. The carrier frequency of Tay-Sachs disease in the non-Jewish population was estimated to be 1/167 using an enzyme-based method (Kaback *et al.*, 1978). That was about twice that (1/300) predicted on the base of the incidence of the disease among non-Jews (Myrianthopoulos and Aronson, 1966). If both of the benign mutations, C739T(R247W) and C745T(R249W), were taken into account in the calculation of the frequency of the enzyme-defined carriers in the general population, the Tay-Sachs disease carrier frequency became 1/280, similar to the 1/300 predicted on the basis of the disease incidence. The remaining difference between the carrier frequency based on the enzyme assay and that estimated from the disease incidence may be accounted for by other benign mutations, such as the putative benigns, G746A(R249Q) (Callahan *et al.*, 1995) and G748A(G250S) (Triggs-Raine *et al.*, 1995).

The diagnostic strategy developed to specifically detect the C745T(R249W) mutation provided Tay-Sachs disease prevention programs with a DNA test to distinguish the carriers of this benign mutation from those who have disease-causing mutations. This procedure could also be used for the detection of the mutation during a prenatal diagnosis if the parents were unavailable for testing, or compound heterozygotes for a benign mutation and a disease-causing mutation. It is now recommended that DNA-testing for the benign C739T(R247W) and C745T(R249W) mutations be done for all carrier couples where one member of the couple is non-Jewish. In many laboratories, all enzyme-defined carriers are tested for the

presence of the C739T(R247W) and C745T(R249W) mutations.

The benign mutations, R247W and R249W, were originally identified in subjects with Hex A pseudodeficiency and they were clearly associated with enzyme deficiency (Kaback *et al.*, 1993; Triggs-Raine *et al.*, 1992; Cao *et al.*, 1993; Tomczak *et al.*, 1993). Although there was close association between these mutations and enzyme deficiency, the possibility was not ruled out that additional mutations in another region of *HEXA* gene, on the same allele, were the cause of the enzyme deficiency. Triggs-Raine *et al.* (1992) had previously shown a substantial level of α-subunit cDNA could be derived from the mRNA isolated from a Hex A pseudodeficient subject. This indicated that the *HEXA* gene with the benign mutation, C739T, was normally transcribed, because the other allele in the subject had the 1278ins4 mutation that did not produce a stable α-subunit mRNA (Boles and Proia 1995).

To determine if the benign mutations, C739T(R247W) and C745T(R249W), were the primary cause of the Hex A pseudodeficiency and if they could be differentiated from disease-causing mutations, we employed a transient expression system using Cos-7 cells to produce the benign mutation-containing Hex S and Hex A. We expressed Hex S because it is an α-subunit dimer and if the benign mutation was present on both subunits in Hex S, their effects might be more obvious than on Hex A. Since Hex S exists in a very small amount *in vivo* and is therefore not physiologically significant, we also expressed mutant Hex A to mimic the physiological form of the enzyme.

Using Cos-7 expression systems, we confirmed the relationship between the benign mutation and Hex A pseudodeficiency. The levels of expressed activity associated with the benign mutation-containing Hex S and Hex A & S were reduced to about 20% to 40% of the

wild type (Table 2 and 3, Fig. 12). A similar level of decrease in the mature α-subunit protein by these benign mutations was also observed (Fig. 13). This is the first direct evidence provided to show that the benign mutations are the primary cause of Hex A pseudodeficiency. Additional evidence leading to the same conclusion had been previously reported. Family studies had shown that the benign mutations were inherited from one of the proband's parents suggesting that they were not randomly acquired mutations (Vidgoff *et al.*, 1973; Kelly *et al.*, 1976; Triggs-Raine *et al.*, 1992; Cao *et al.*, 1993). Further, population studies demonstrated that the benign mutations were found only in the enzyme-defined carriers, but not in non-carriers and obligate disease carriers (Triggs-Raine *et al.*, 1993; Kaback *et al.*, 1992; Cao *et al.*, 1993).

Using the Cos-7 expression system, we also demonstrated that the benign mutations could be differentiated from the mutations associated with adult-onset and other forms of $G_{M2}$ gangliosidosis. It was clearly shown that the decrease in the levels of Hex S and Hex A & S activity and of α-subunit protein due to the benign mutations were much less significant than the reduced levels resulting from even the mildest disease-causing mutation, G269S (Fig.12 and 13). Normally there is an overlap between the percent Hex A activity that is associated with the various phenotypes of this disease, including pseudodeficiency, when Hex A activity is determined from serum, leucocytes, or fibroblasts (Kelly *et al.*, 1976; Thomas *et al.*, 1982; Grebner *et al.*, 1986; Navon *et al.*, 1986). The results from these studies extended a previous study by Brown and Mahuran (1993) in which they showed that α-/ß-subunit co-expression could distinguish the adult-onset mutation from those associated with more severe forms of $G_{M2}$ gangliosidosis.

In our expression studies, the disease-causing mutations resulted in a lower percent Hex A & S, compared to normal, than that previously reported for α-/ß-subunit co-expression (Brown and Mahuran, 1993). This may be because (1) whole cell extracts were used, instead of immunoprecipitated enzyme, to determine Hex A & S activity, and (2) the differences in the ratio of αpSVL/pCD43, the DNA concentration, and the method used for DNA transfection.

In our expression system, the conditions for transfection, aimed at expressing Hex S and Hex A above the endogenous level of ß-hexosaminidase activity in Cos-7 cells, were determined by measuring the expressed Hex A & S activity using 4-MUG as the substrate (Fig. 3 to 8). The conditions for co-transfection of the α- and ß-subunit cDNAs, αpSVL and pCD43, into Cos-7 cells that we chose to express Hex A (αß), were also found to result in a significant level of Hex S (αα) in addition to Hex A and B (ßß) (Fig.15). This indicated the conditions we chose for Hex A expression were not fully optimized. This might be because only the levels of heat-sensitive activity towards the substrate 4-MUG were used to determined the level of α-subunit dimers; this could not distinguish Hex A activity from Hex S activity. Consequently , we use the term Hex A & S throughout most of the sections to describe Hex A expression, because an elevated Hex S activity is present, in addition to Hex A, in the transfected Cos-7 cells. Hex S cannot be readily differentiated from Hex A using 4-MUG or 4-MUGS as a substrate. Further, when αpSVL was transfected alone into Cos-7 cells, both Hex S and a substantial amount of Hex A were formed (Fig.16) indicating that dimers between expressed human α-subunits and the endogenous Cos-7 ß-subunits were formed. In this case, the term Hex S is still used to differentiate it from Hex A. Therefore,

the activity of Hex S expressed in Cos-7 cells reflected combined activities of expressed human Hex S and the hybrid (ie. monkey and human) Hex A, even though the expressed Hex S was predominant.

To normalize the transfections, we co-expressed ß-galactosidase with the α-/ß-subunit and measured the expressed ß-galactosidase activity. The levels of expressed Hex A & S activity were normalized using the expressed level of ß-galactosidase activity. However, the level of normalized Hex S and Hex A & S activities was found to be influenced by the transfection efficiency. When transfection efficiency was low, the mutant enzymes had a higher ß-hexosaminidase activity compared to the normal, i.e. wild type, level, and a higher level of α-subunit protein. Higher transfection efficiencies resulted in lower percent Hex A & S and correspondingly lower levels of α-subunit protein. As seen in Fig.13, panel B, mutant Hex A & S containing the benign mutations showed higher expressed levels of the α-subunit protein in this experiment where the transfection efficiency was lower than those shown in Fig.13, panel C.

Data derived from the current studies and previous studies suggest that benign mutations should not cause disease. The Hex A levels in fibroblasts and leucocytes of Hex A pseudodeficient subjects with the R247W and R249W mutations (Kelly *et al.*, 1976; O'Brien *et al.*, 1978; Vidgoff *et al.*, 1973; Thomas *et al.*, 1982; Grebner *et al.*, 1986; Navon *et al.*, 1986; Triggs-Raine *et al.*, 1992; Cao *et al.*, 1993) are usually slightly higher than that which is found in a patient with juvenile or adult-onset $G_{M2}$ gangliosidosis (Navon *et al.*, 1986). Results from *in vitro* and *in situ* $G_{M2}$ ganglioside hydrolysis assays (Thomas *et al.*, 1982; Grebner *et al.*, 1986), together with the results derived from the Cos-7 expression

system, suggest that the level of the enzyme associated with benign mutations is above the critical threshold required for normal $G_{M2}$ ganglioside turnover. Indeed, several pseudodeficient subjects are more than 40 years old (Triggs-Raine *et al.*, 1992; Cao *et al.*, 1993), and patients with the adult-onset mutation G805A(G269S) typically show symptoms before the age of 40 years (Navon *et al.*, 1986 and 1990). However, one cannot rule out the possibility of a late-onset phenotype in these subjects.

In our studies, we used Hex A pseudodeficient fibroblasts in addition to the transient expression system. The use of fibroblasts from Hex A pseudodeficient subjects provided a more realistic system than the Cos-7 expression system for the analysis of mutant ß-hexosaminidase because it reflects the natural *in vivo* process for mutant Hex A expression rather than the amplified mutant Hex A & S in Cos-7 cells. However, use of the Cos-7 expression system also has some advantages: (1) It can produce mutant enzyme without interference from the expression of a second allele as occurs in fibroblasts, (2) the amplified signals can provide a hint about the benign mutation's effect on Hex A which can not be seen in the fibroblasts, and (3) the fibroblasts are not available from a genetic compound for the C745T(R249W) mutation and a disease-causing mutation.

Hex A & S from transfected Cos-7 cells and Hex A from fibroblasts were characterized to determine the basis of the reduction in Hex A activity by the benign mutations.

Several characteristics of the enzyme including pH optimum, heat sensitivity, $K_m$ and $V_{max}$ were assessed. The pH optimum for the reaction catalyzed by Hex A & S carrying either of the benign mutations remained unchanged (Fig.20 and 21), indicating that the R247 and

R249 residues are not required for proton transfer in the catalytic reaction and are not involved in the catalytic reaction. Interestingly, when the residue Asp258 in exon 7 of the α-subunit, which locates in the pocket involved in substrate binding and catalytic reaction, was substituted with the amino acid histidine, Hex A's optimal pH for catalytic reaction was changed (Bayleran *et al.*, 1987; Fernandes *et al.*, 1992b).

The Hex A & S activity remaining after a treatment at 45°C or 50.1°C for 90 and 120 min was the same for both the normal Hex A & S and that carrying the C739T(R247W) or C745T(R249W) mutation (Fig.18 and 19). The results of heat-inactivation tests are influenced by inaccuracies in the reaction time, incubation temperature, and by the amount of total protein in the reaction mix. Although these factors were tightly controlled in our experiments, small differences in heat sensitivity may still be missed. The Hex A activity remaining in normal and pseudodeficient fibroblasts after a treatment at 37°C also found to be similar in studies by Grebner *et al.* (1986). However, at 45°C, we found the G269S (adult-onset mutation) containing Hex A & S was more heat-sensitive than normal Hex A & S (Fig.17). This was consistent with the finding of Brown and Mahuran (1993). This suggested that the benign mutations do not increase the heat sensitivity of the α-subunit *in vitro*.

To further characterize Hex A containing the benign mutations, Hex A was separated from Hex B and Hex S and used to study the kinetics of the mutant enzyme. The isoenzyme separation was performed because a substantial amount of Hex S was formed by the co-transfection of pCD43 and αpSVL into Cos-7 cells using the conditions predicted by our initial studies to produce Hex A. The ß-hexosaminidase activity profile derived from the

column chromatography used for Hex A isolation once again showed that the transfection conditions were not optimal for Hex A expression. Under the conditions used for transfection (see section 2.14.1 and 3.1.0), Hex A was still predominant, although there was a significant level of Hex S produced (Fig.15).

Hex A harbouring either of the benign mutations, C739T(R247W) or C745T(R249W), exhibited no significant change in the $K_m$ (Table 5) suggesting that the benign mutations did not affect Hex A's substrate binding affinity or specificity for the synthetic substrates. The basis for the apparent reduction in the $V_{max}$ of Hex A by the benign mutations was the decrease in the α-subunit protein. This was demonstrated by showing that the quantity of normal and C739T(R247W)- or C745T(R249W)-α-subunit protein of mature size producing equivalent levels of activity toward the synthetic substrate appeared similar on western blot analysis (Fig.23).

Our studies then focused on the basis of the decrease in the protein level of the mature α-subunit harbouring the benign mutations. It had previously been shown that fibroblasts from Hex A pseudodeficient subjects synthesized an apparently normal amount of the precursor α-subunit, which was then normally processed to its mature form and normally secreted extracellularly (Thomas *et al.*, 1982; Grebner *et al.*, 1986). To confirm the previous observations, we also followed the biosynthetic pathway of Hex A in fibroblasts from Hex A pseudodeficient subjects by pulse-chase analysis of metabolically labelled proteins. We first determined if the benign mutations affect the biosynthesis of the α-subunit. A normal level of precursor α-subunit was observed in both the pulse-labelled Cos-7 cells transfected with the benign mutation containing α-subunit cDNA (Fig.27) and the fibroblasts from Hex A

pseudodeficient subjects (Fig.28 and 29). This suggested that the benign mutations did not affect α-subunit synthesis.

Our next step was to determine if the benign mutations affected the phosphorylation of the α-subunit. The benign mutation-containing fibroblasts showed a radioactive signal corresponding to the precursor α-subunit after the cells were pulse-labelled with $^{32}P$ (Fig.25). The same radioactive signal was missing in Tay-Sachs disease fibroblasts indicating that the α-subunit containing the benign C247T (R247W) mutation was normally phosphorylated. Unfortunately, the signal in these experiments was weak, and the background levels were high, making it difficult to compare the levels of the phosphorylated α-subunit between normal and pseudodeficient fibroblasts.

The phenomenon of nonuniform Hex A deficiency in tissues and fluids of Hex A pseudodeficient subjects containing the benign mutation C739T(R247W) indicated that this mutation might affect the secretion of Hex A (Thomas *et al.*, 1982, Grebner *et al.*, 1986, Navon *et al.*, 1986; Triggs-Raine *et al.*, 1992). We performed experiments to determine if Hex A containing the benign R247W mutation could be normally secreted. The Hex A pseudodeficient fibroblasts were grown in the presence of $NH_4Cl$. When $NH_4Cl$ is taken up, the pH value in the lysosomal compartment is elevated and the dissociation of Hex A from the mannose-6-phosphate receptor is prevented (Seglen, 1983). As a result, Hex A is forced to the secretory pathway. The secretion of Hex A with the benign mutation was clearly enhanced by the $NH_4Cl$ treatment (Fig.26) indicating that: (1) the α-/ß-subunit dimer with the benign mutation in the α-subunit is not trapped in the endoplasmic reticulum and the Golgi apparatus, (2) the benign mutations do not affect the secretion of Hex A from fibroblasts, and

(3) the mutant Hex A can be normally delivered to the lysosome, or prelysomal compartments, where the effect of the $NH_4Cl$ took place.

The basis for the reduced level or absence of Hex A in the serum of R247W mutation-containing Hex A pseudodeficient subjects, as summarized by Grebner *et al.* (1986), is currently unknown. It may be that this benign mutation blocked Hex A's secretion, increased Hex A's lability in the serum, or increased Hex A's clearance from the blood system. The second possibility is less likely, because Hex A containing the R247W mutation had normal heat-sentivity when compared to normal Hex A in both fibroblasts and transfected Cos-7 cells (Fig.18 and 19; Grebner *et al.*, 1986). Even though Hex A containing the R247W mutation showed normal secretion from fibroblasts in our studies, an effect of this benign mutation on Hex A secretion could not be excluded since the tissue origin of serum Hex A remains unknown.

Further analysis of the benign mutation-containing α-subunit by pulse-chase experiments using both transfected Cos-7 cells and the fibroblasts from Hex A pseudodeficient subjects revealed a decrease in α-subunit stability. In the Cos-7 cells, only the normal and mutant α-subunit cDNAs were introduced to express Hex S. In comparison with normal Hex S, the benign mutation-containing Hex S showed a reduced level of the mature α-subunit at different chase times, and a slightly elevated level of the precursor α-subunit (Fig.27). This may reflect a delay in the precursor conversion to its mature form during the chase period from 2 hr to 14 hr (Fig.27). However, at 20 hr chase, the α-subunit precursor containing the benign mutations was similar in abundance to that of the normal. This suggested that the two benign mutations affect the stability of Hex S in the lysosome,

and possibly also the early processing of the α-subunit, leading to degradation of accumulated precursor. The adult-onset disease causing mutation, G805A(G269S), is thought to affect the α-subunit folding and stability, therefore indirectly affecting the formation of α-/ß-subunit dimers (Brown and Mahuran, 1993). When this mutation was compared with the benign mutations in the pulse-chase experiments, it showed a very small amount of the mature α-subunit and an unstable precursor α-subunit that disappeared by 20 hr of chase (Fig.27). Since the benign mutation-containing α-subunit precursor was still present at 20 hr of chase, this suggested that its effects on α-subunit folding may exist, but if so, they were very mild.

We also designed our experiments differently from those by Thomas *et al.* (1982) and Grebner *et al.* (1986) to test the α-subunit stability in Hex A pseudodeficient fibroblasts. In our experiments, we used equal amounts of protein from the cell extracts and three types of antibodies, anti-α-subunit, anti-Hex B and anti-Hex A for immunoprecipitation. To avoid the interference from partially processed forms of the ß-subunit that migrate on gels in the same position as the mature α-subunit, we also developed a protocol using the anti-α-subunit antibody to specifically immunoprecipitate the α-subunit in free and ß-subunit associated forms.

In our pulse-chase experiments in fibroblasts from Hex A pseudodeficient subjects, a reduced level of mature α-subunit was shown (Fig.28 and 29) that corresponded to a previous report (Grebner *et al.*, 1986); but the elevated level of the α-subunit precursor observed when the benign mutation-containing α-subunit was expressed as Hex S in Cos-7 cells was not seen. Instead, the α-subunit precursor was seen to constantly decrease. The different observations in the two expression systems might result from the overexpression of

Hex S in Cos-7 cells; the over-expressed signal might provide information that may not be seen naturally. The results derived from these studies using both Cos-7 and fibroblast expression systems and from the previous studies (Grebner *et al.*, 1986) consistently showed a reduced protein level or stability of the mature α-subunit. This observation was further substantiated by using two procedures to immunoprecipitate the α-subunit (Fig. 28 and Fig.29). The fibroblast studies using the α-subunit specific antibody also showed a constant decrease in α-subunit precursor suggesting that prelysosomal stability may also be affected.

Using lysosomal protease inhibitors, we were unable to confirm that benign mutations affect the α-subunit processing and stability by stabilizing the proteins. The addition of Leupeptin and E-64, inhibitors of some lysosomal proteases (Seglen, 1983), had no effect on the level of mature α-subunit protein and Hex A activity (Table 4; Fig.30 and 31). The benign mutations may render the α-subunit susceptible to a lysosomal protease(s) that cannot be inhibited by Leupeptin or E-64. Alternatively, the mutant α-subunit may be susceptible to non-enzymatic factors. The decreased level of mature α-subunit protein is consistent with the hypotheses of Grebner (1986) and Thomas (1982), that these mutations reduce the enzyme level and therefore the activity toward both the synthetic and natural substrates.

The residues, R247 and R249, which are mutated in individuals with Hex A pseudodeficiency, fall in a highly conserved region of exon 7 of the human α- and ß-subunits (Myerowitz *et al.*, 1985; O'Dowd *et al.*, 1985; Korneluk *et al.*, 1986), mouse α- and ß-subunits (Bapat *et al.*, 1988; Beccari *et al.*, 1992), boar ß-subunit (unpublished, Genbank # X92379), yeast ß-hexosaminidase (Cannon et al., 1994), and a region of ß-hexosaminidase from *Dictyostelium discoideum* (Graham *et al.*, 1988), ß-hexosaminidase of *Streptomyces*

*plicatus* (Triggs-Raine *et al.*, unpublished data), *Vibrio vulnificans* (Somerville and Colwell, 1993) and chitobiase of *Vibrio harveyi* (Soto-Gil and Zyskind, 1989) (Fig.1). This suggests that these residues are important in maintaining normal Hex A function. A predicted chitobiase structural model (Tews *et al.*, 1996) suggested that these two residues, R247 and R249, are on the outside of the subunit. They might be involved in maintaining stability of the α-subunit and/or in interacting with the ß-subunit.

# 5. CONCLUSION AND FUTURE DIRECTIONS

In the current studies, the frequency of the second benign mutation, C745T(R249W), was estimated to be about 6% of enzyme-defined carriers in non-Jewish population. The benign mutations, C739T(R247W) and C745T(R249W), were confirmed to be the primary cause of Hex A pseudodeficiency and they can be differentiated from the disease-causing mutations using transient expression system. The benign mutations reduce Hex A activity by affecting the level of the α-subunit protein, not by affecting Hex A's affinity and / or specificity to the synthetic substrates, 4-MUG and 4-MUGS, not by affecting the Hex A's catalytic site, and nor by increasing its heat sensitivity. The reduced protein level of the α-subunit by the benign mutations resulted from *in vivo* α-subunit protein instability.

In the future, some remaining questions concerning Hex A pseudodeficiency need to be investigated. The objectives to answer these questions are outlined below.

(1) To quantitatively analyze the ability of Hex A from Hex A pseudodeficient subjects to hydrolyse $G_{M2}$ ganglioside. The hypothesis for this is that the reduced protein level of the α-subunit by benign mutations produces Hex A activity at a level just above the critical threshold required for normal $G_{M2}$ ganglioside hydrolysis. Although, a normal level of $G_{M2}$ ganglioside hydrolysis was shown in the Hex A pseudodeficient fibroblasts (Grebner *et al.*, 1986; Triggs-Raine *et al.*, 1992), their activity measured by *in vitro* assays might not be specific to Hex A since detergent was used in the reaction and the *in situ* assays were not quantitative. An analysis using a $G_{M2}$ analogue showed a Tay-Sachs disease carrier level of Hex A activity in Hex A pseudodeficient subjects (Agmon *et al.*, 1996); however, analysis using the natural substrate $G_{M2}$ ganglioside and $G_{M2}$ activator protein would provide more convincing results. In these studies, although the activities of Hex A & S and Hex S were

determined using the synthetic substrates, 4-MUGS or/and 4-MUG, these data suggested that the C739T(R247W) and C745T(R249W) mutations would produce a level of Hex A with activity above the critical threshold required for normal $G_{M2}$ ganglioside hydrolysis (Leinekugel *et al.*, 1992).

(2) To follow up the biological data and neurological examinations of the Hex A pseudodeficient subjects. If they developed late-onset neurological disorders, other neurological diseases would be ruled out. Hex A activity and the level of accumulated $G_{M2}$ ganglioside would be determined to confirmed the diagnosis.

(3) To determine if the two putative benign mutations, G748A(G250S) and G746A(R249Q), are benign mutations. Hex S and Hex A & S containing one of the putative benign mutations would be expressed in Cos-7 cells. The expressed activities could be compared with the normal, benign mutation and disease-causing mutation-containing Hex S and Hex A & S.

(4) To determine the tissue origin of serum Hex A in order to understand the biology of the secreted Hex A. This would also be helpful in determining if the benign mutations affect Hex A secretion and provide a reason for the nonuniform Hex A deficiency in the tissue and fluids in R247W-containing Hex A pseudodeficient subjects. Tissue specific gene targeting experiments in the newly developed Tay-Sachs disease mice may be useful for this study.

(5) To determine if the benign mutations have an effect on the α- and ß-subunit interaction. The predicted location of the residues, R247 and R249 (Tews *et al.*, 1996), on the α-subunit suggests they could be involved in maintaining the stability of the α-subunit and

potentially in interacting with the ß-subunit. Our studies demonstrated that benign mutations affect the level of the α-subunit by making it less stable. Although the α-subunit containing the benign mutations can form active αα and αß dimers, the benign mutations may affect the affinity for the α-subunit for the ß-subunits, so that the association was partially affected.

(6) To determine the stability of precursor α-subunit harbouring the benign mutations. The unstable precursor α-subunit containing the benign mutations was observed in the transfected Cos-7 cells, though not seen in fibroblasts in our studies. This indicated that the benign mutations may have an effect on the α-subunit at an early processing stage. In the experiments for testing for this hypothesis, normal and Hex A pseudodeficienct fibroblasts will be treated with Brefeldin A (Boehringer Mannheim), an inhibitor that can block the protein translocation process from the endoplasmic reticulum to the Golgi apparatus. The level of α-subunit precursor will be determined by pulse-chase analysis and immunoprecipitation.

(7) To determine if the benign mutations have the effect on the folding of the α-subunit. Hex A pseudodeficient fibroblasts will be grown at 37°C or/and reduced temperature (i.e. room temperature) in the α-MEM supplemented with antibiotics and fetal bovine serum in the presence of glycerol. If the benign mutation affects the folding, higher levels of both mature and precusor α-subunit protein will be obtained with glycerol in the cell culture medium than that without glycerol at the reduced temperature or at both 37°C and the reduced temperature.

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