

Metachromatic Leukodystrophy: the Role of Non-Pathogenic Sequence Variants in the Causation of Disease

by

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ERRATUM

Throughout the text of this thesis, microlitre should be abbreviated as " μ l" and not as "ul"

page 9, line 6;

"....Austin (1959) and independently showed that....", should read "....Austin (1959) showed that...."

page 25, line 17;

"....numerous of other authors....", should read "....numerous other authors...."

page 26, line 21;

"....a patient will present conversely, disease....", should read "....a patient will present. Conversely, disease...."

page 57, line 21 and page 107, line 9;

"-" and "20" (for temperature) should not be separated by a space.

page 127, line 1;

"4.3.3 The regulation of steady state ASA mRNA levels", should read

"4.4 CONCLUSIONS"

"4.4.1 The regulation of steady state ASA mRNA levels"

page 129, line 4;

"4.3 CONCLUSIONS", should read **"4.4.2 Concluding Remarks"**.

page 139, lines 2 and 3;

"....The ASA cDNA 5' untranslated sequence....", should read "....The ASA cDNA and the 5' untranslated sequence....".

page 202, line 20;

"....variety different intragenic....", should read "....variety of different intragenic....".

I think it's the most extraordinary collection of talent, of human knowledge, that has ever been gathered together at the White House - with the possible exception of when Thomas Jefferson dined alone.

John Fitzgerald Kennedy (1917-1963)

Said at a dinner of Nobel Prizewinners, 29th April 1962

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ABSTRACT

Metachromatic leukodystrophy (MLD) is a lysosomal storage disorder caused by a deficiency of the enzyme arylsulphatase A (ASA). ASA is responsible for the degradation of cerebroside sulphate, a sphingolipid found as a major component of myelin. A deficiency of ASA leads to the accumulation and lysosomal storage of cerebroside sulphate within nerve cells, causing progressive demyelination of the central and peripheral nervous system.

MLD is diagnosed biochemically by the measurement of residual ASA activity in cultured skin fibroblasts or blood leukocytes. Diagnosis is, however, complicated by the presence of individuals in the normal population with ASA activities in the range of MLD patients. This non-pathogenic deficiency of ASA has been termed 'ASA pseudodeficiency' (ASA-PD) and is commonly caused by a 90% reduction in ASA mRNA levels and hence ASA protein, leaving pseudodeficient individuals with only 10% of normal ASA activity. Approximately 1% of the normal population are homozygous for the ASA-PD allele. The existence of clinically normal individuals with only 10% of normal ASA activity suggests that most ASA enzyme that is produced is far in excess of that required for the normal hydrolysis of cerebroside sulphate. The threshold level of ASA activity which separates normal development from the development of MLD has been estimated in this thesis to be between 3% and 4% of normal.

Eight ASA mutations had been identified prior to the commencement of this project. Our MLD patient population (N=29) was screened for the presence of each of these alleles to identify a subset of 13 patients in which no known pathogenic mutations were identified. Single strand conformation polymorphism and sequence analysis identified a further nine novel putative MLD mutations within this group.

In some cases there was a clear correlation between genotype and clinical presentation, for example, a single mutation, T274M, was found to account for the presentation of six MLD patients of Lebanese descent. However, numerous patients were found to have complex ASA alleles containing multiple sequence variants. The role that these multiple alterations played in the development of MLD was unclear, particularly in view of the fact that when a number of these sequence variants were expressed *in vitro*, they were found to reduce ASA activity without causing MLD.

The individual expression of each ASA sequence variant and the expression of complex patient alleles has led to a greater understanding of the role that non-pathogenic changes within the ASA gene can play in the development of MLD. Specifically this work has demonstrated that;

- 1) Individually non-pathogenic sequence variants in the ASA gene can cause MLD in the absence of recognised MLD mutations when they are combined on a single allele.
- 2) When non-pathogenic sequence variants are found in combination with mutations causing mild MLD, they can act to modify the expression of disease causing mutations and increase disease severity.
- 3) Non-pathogenic sequence variants can have different effects on phenotype depending on the background level of ASA activity on which they are expressed.

The definition of a role for non-pathogenic sequence variants in the causation of MLD has significant consequence for the development and presentation of other lysosomal and non-lysosomal disorders.

**They are ill discoverers that think there is no land,
when they can see nothing but sea.**

Francis Bacon (1561-1626)

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Signed:

John S. Harvey

October 6, 1996

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I wish to dedicate this thesis to my best friend and now, thankfully, wife Sharon (Doc) Orford for her love and support over the many years that have lead to the submission of this document. Some debts can never be repaid, only graciously acknowledged.

Life moves pretty fast, if you don't stop and look around once in a while, you could miss it. Thanks everyone, its been a blast !

Dum vivimus vivamus, ergo bibamus.

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LIST OF ABBREVIATIONS

A	adenine
A ₂₆₀	optical density at 260 nm
A ₅₁₅	optical density at 515 nm
A ₆₀₀	optical density at 600 nm
ASA	arylsulphatase A
<i>ASA</i>	the arylsulphatase A gene
ASO	allele specific oligonucleotide
[α - ³² P]dCTP	alpha-labelled deoxycytosine triphosphate
[α - ³² P] UTP	alpha-labelled uridine triphosphate
BMT	bone marrow transplant
bp, kb	base pairs, kilobase pairs
C	cytosine
cDNA	deoxyribonucleic acid complementary to ribonucleic acid
CHO cell	Chinese hamster ovary cell
cm, μ m	centimetre(s), micrometre(s)
CNS	central nervous system
cpm	counts per minute
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytosine-5'-triphosphate
dH ₂ O	distilled water
dGTP	2'-deoxyguanosine-5'-triphosphate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
DTT	dithiothreitol
dTTP	2'-deoxythymidine-5'-triphosphate
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
EtBr	ethidium bromide
EtOH	ethanol
g	force of gravity
G	guanine
g, mg, μ g, ng,	gram(s), milligram(s), microgram(s), nanogram(s).
[γ - ³² P]dATP	gamma-labelled adenosine triphosphate

GAPDH	glyceraldehyde 3-phosphate dehydrogenase
H ₂ O	distilled water
hr/min/sec	hour(s)/minute(s)/second(s)
IPTG	isopropyl-β-D-thiogalactoside
kDa	kilodaltons
kPa	kilopascal
L, ml, ul	litre(s), millilitre(s), microlitre(s)
M	moles per litre
mCi	microCuries
MLD	metachromatic leukodystrophy
MOPS	3-(N-Morpholino)propanesulphonic acid
mRNA	messenger ribonucleic acid
N-terminal	amino-terminal
°C	degrees Celsius
PCR	polymerase chain reaction
PD	pseudodeficiency
PEG	polyethylene glycol
poly(A)	polyadenylylated
rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
RNaseA	ribonuclease A
RNaseT1	ribonuclease T1
rpm	revolutions per minute
rRNA	ribosomal RNA
SDS	sodium dodecyl sulfate
SSC	saline sodium citrate
T	thymidine
<i>Taq</i>	<i>Thermus aquaticus</i>
TEMED	NNN'N'-Tetramethylethylenediamine
Tris	Tris[hydroxymethyl]amino methane
U	unit(s) of enzyme
UV	ultraviolet
V	Volts
X-gal	5'-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
% (v/v)	percent volume per volume
% (w/v)	percent weight per volume

Chapter 1

Introduction

1.1 THE LYSOSOMAL STORAGE DISORDERS



The concept of an inborn error of metabolism was first introduced by Garrod in 1908 when he perceived a relationship between genes and enzymes. Hundreds of genetic disorders which result from the deficiency of enzymes in a metabolic pathways have subsequently been identified and are comprehensively reviewed in Scriver *et al.* (1995).

Lysosomes are cytoplasmic, acidic vacuoles which contain a range of enzymes which are involved in the degradation of a variety of cellular macromolecules (Kornfeld, 1992). The common feature of the lysosomal enzymes is that they are all broadly involved in catabolic pathways and either degrade specific macromolecules or play a role in the transportation of these molecules within the cell (Gieselmann *et al.*, 1991). The lysosomal storage disorders are a group of 36 inborn errors of metabolism which are characterised by the deficiency of one or more of these lysosome-associated enzymes (Gieselmann *et al.*, 1995).

A deficiency of a lysosomal enzyme causes the storage of the natural substrate of the enzyme within the lysosome and eventually leads to the malfunction of the organ in which the storage occurs. The concept of a lysosomal storage disorder was introduced by Hers (1965), to explain the link between a deficiency of the enzyme α -glucosidase and Pompe disease (Neufeld, 1991). The natural substrate of α -glucosidase, glycogen, was found to gradually accumulate within the lysosomes of patients with Pompe disease and resulted in an increase in the size and number of these organelles within the cell. The storage of substrate lead to the inhibition of cellular and organ function and to the presentation of the disease.

The lysosomal enzymes can be broadly grouped into classes according to the degradation pathway in which they involved and the specific substrate that is accumulated when they are deficient. There is some overlap between these groups because some lysosomal enzymes are specific for terminal residues which are common to more than one substrate. However, ten enzymatic deficiencies lead to the accumulation of

mucopolysaccharides, five to the accumulation of glycoproteins and remaining eight enzymes are broadly involved in the metabolism of sphingolipids (Gieselmann *et al.*, 1995). The deficiency of one or more enzymes involved in sphingolipid degradation results in the presentation of a group of lysosomal storage disorders known collectively as the lipidoses.

1.2 SYNTHESIS AND TARGETING OF THE LYSOSOMAL HYDROLASES

1.2.1 Synthesis and Post-Translational Modification

The synthesis, post-translational processing and cellular localisation of the lysosomal enzymes has been reviewed in detail by numerous authors (von Figura and Hasilik, 1986; Neufeld, 1991; Kornfeld, 1992).

All proteins, whether they are destined for the cell surface, storage granules or the lysosome, initially share a common synthetic pathway. N-terminal signal peptides allow the targeting and internalisation of proteins in the endoplasmic reticulum (ER) (Figure 1.1) (Pfeffer, 1988). Within the ER, enzymes that are destined for the lysosome have high mannose oligosaccharides incorporated onto various glycosylated asparagine residues within their peptide sequences. The asparagine residues that are glycosylated are found in an Asn-X-Thr/Ser consensus sequence, although not all of these potential N-glycosylation amino acid sequence sites are used. The N-linked oligosaccharide side chains are trimmed within the ER before the proteins are transported to the Golgi in membrane-bound vesicles (Figure 1.1). Within the Golgi the mannose residues on the N-linked side chains are modified by the action of glycosidase and phosphorylated by a phosphotransferase (Kornfeld and Kornfeld, 1985; Pfeffer, 1988). It is the acquisition of the phosphorylated mannose residues (mannose 6-phosphate) that directs proteins to the lysosome by their interaction with specific receptors in the trans-Golgi body.

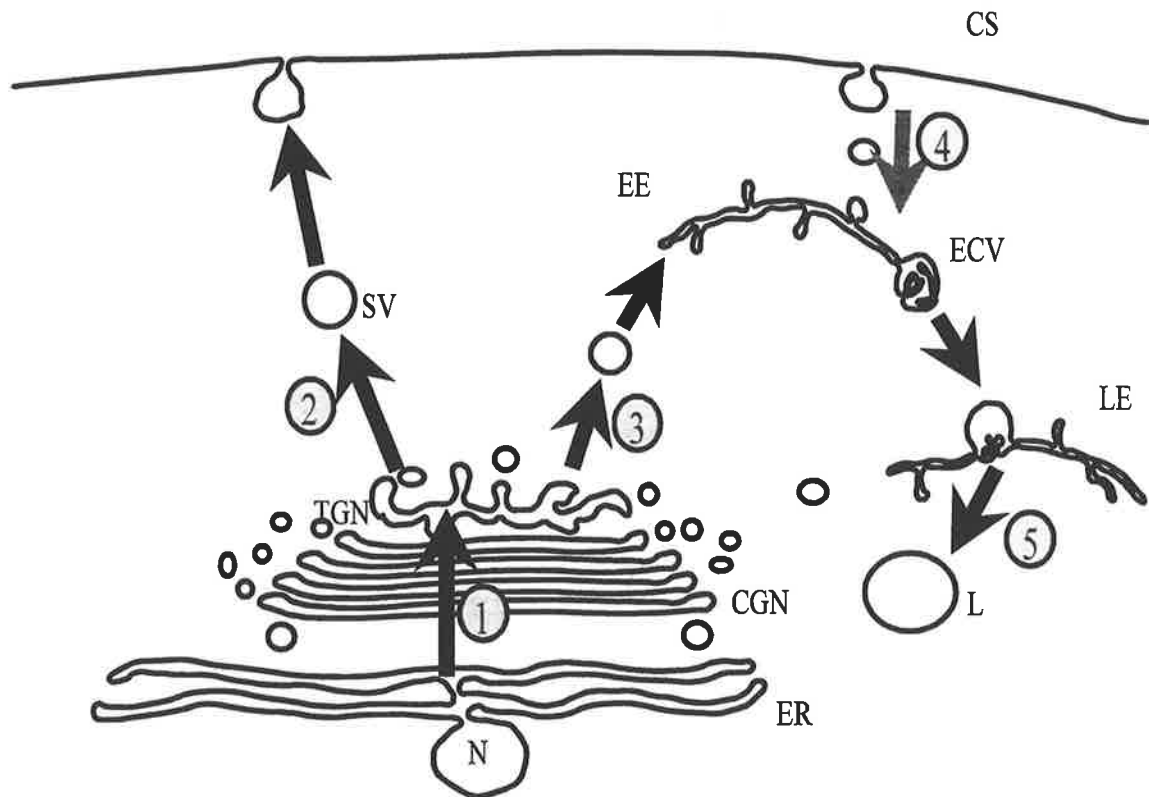
1.2.2 Subcellular Transport

Two distinct mannose 6-phosphate (M6P) receptors of 300 kDa and 46 kDa have been identified. Both specifically bind the oligosaccharides bearing phosphomannosyl groups on the lysosomal enzymes in the trans-Golgi and subsequently release the enzymes in response to the acidic environment of the lysosome. Despite the similarity in function of the two receptors, they do not display a high degree of sequence similarity, apart from a 145 amino acid region of cysteine rich repeat units which, whilst constituting an important structural motif, do not define the mannose 6-phosphate binding capacity of the receptors.

The enzyme/receptor complex exits the trans-Golgi network in clathrin-coated vesicles although this complex does not reach the lysosome intact since the receptor can not be identified in mature lysosomes (Figure 1.1). The Golgi vesicles fuse with acidic prelysosomal (or early endosomes) compartments where the low internal pH of the compartment facilitates the release of the enzyme (Figure 1.1) from the receptor which then returns to the Golgi complex for further enzyme transportation. The targeting of enzymes to the lysosome *via* mannose 6-phosphate receptors is not however 100% efficient and a fraction of newly synthesised lysosomal enzymes are secreted (Neufeld, 1991). Mannose 6-phosphate receptors found on the cell surface are involved in the internalisation of these exogenous lysosomal enzymes (Figure 1.1).

Within the lysosome, enzymes undergo further processing which includes the dephosphorylation of mannose 6-phosphate. The acidic environment of the lysosomes not only facilitates the disassociation of the enzyme/receptor complex but also provides an optimal environment for the action of the lysosomal enzymes upon their substrates.

The importance of the mannose 6-phosphate receptors in targeting enzymes to the lysosome is illustrated by patients with I-cell disease. The lysosomal enzymes of I-cell patients



Adapted from Hopwood and Brooks (1996)

Figure 1.1 Synthesis and cellular targeting of lysosomal enzymes

Schematic representation of the synthesis and post-translational targeting of lysosomal enzymes. ER: endoplasmic reticulum; N: nucleus; SV: secretory vesicle; EE: early endosome; LE: late endosome; L: lysosome; TGN: trans-Golgi network; CGN: cis-Golgi network; CS: cell surface; ECV: endocytic vesicle

- 1) Transportation of proteins from the ER to the CGN via membrane bound vesicles.
- 2) Secretion of mistargeted enzymes from the TNG to the cell surface in secretory vesicles.
- 3) Transportation of the lysosomal enzyme/M6P receptor complex to the EE.
- 4) Internalisation of exogenous lysosomal enzymes via cell surface M6P receptors.
- 5) Maturation of late-endosomes into lysosomes.

fail to acquire mannose 6-phosphate due to a phosphotransferase deficiency. The enzymes are therefore not recognised by the mannose 6-phosphate receptors and consequently, I-cell patients have barely detectable levels of the lysosomal hydrolases. However, large amounts of these proteins circulate in plasma and are excreted in urine which indicates that they are processed by default, through the secretory pathway. An important observation is that not all lysosomal enzymes are deficient in I-cell patients, which indicates the existence of mannose 6-phosphate-independent trafficking of enzymes to the lysosome. Some lysosomal membrane associated proteins, such as β -glucocerebrosidase, are targeted to the lysosome in a manner which is independent of the presence of N-linked phosphorylated mannose residues (Barriocanal *et al.*, 1986).

1.3 METACHROMATIC LEUKODYSTROPHY

1.3.1 Introduction

Metachromatic leukodystrophy (MLD) is an autosomal recessive inborn error of metabolism caused by a deficiency of the lysosomal hydrolase, arylsulphatase A (ASA). The disorder has a population frequency of approximately 1 in 40 000 live births (Gustavson and Hagberg, 1971). A deficiency of ASA leads to the lysosomal accumulation of the natural substrate, cerebroside sulphate, which is a major component of myelin (Norton and Poduslo, 1973), within nervous tissue and the onset of characteristic symptoms.

MLD is clinically characterised by ataxia, dementia, optic atrophy and progressive spastic quadraparesis. Variation in the age of onset provides the basis of classification into three disease subtypes, late-infantile, juvenile and adult. Disease severity appears to be governed by the degree of enzyme deficiency; the greater the deficiency of ASA, the earlier the age of onset of the disorder and the faster its progression (reviewed by Kolodny and Fluharty, 1995). The name of the disorder is derived from the distinct 'metachromatic'

staining produced when nervous tissue from MLD patients is treated with acid-cresyl stains which differentiate the lysosomes storing excess cerebroside sulphate. 'Leuko' refers to white as in white matter and 'dystrophy' to a degeneration. Leukodystrophy therefore implies a degeneration of white matter.

Following the isolation of the ASA gene (Stein *et al.*, 1989) and its localisation to the long arm of chromosome 22 at 22q13, a large number of MLD causing mutations have been characterised (reviewed by Gieselmann *et al.*, 1994) and there appears to be a good correlation between the nature of the mutations, the presence or absence of residual enzyme activity and the severity of disease observed in patients. However, not all deficiencies of ASA lead to the development of MLD. Healthy individuals with about a ten-fold reduction of ASA activity levels are observed in the population with a frequency of approximately 1%. Since this low level of activity is obviously sufficient to prevent substrate accumulation and disease symptoms, these individuals are said to have ASA pseudodeficiency (ASA-PD).

A number of functionally related mammalian and non-mammalian sulphatase enzymes have been isolated and sequenced (Peters *et al.*, 1990; Yen *et al.*, 1987; Sasaki *et al.*, 1988; Yang *et al.*, 1989; Murphy *et al.*, 1992; Tomatsu *et al.*, 1991; Wilson *et al.*, 1990; Robertson *et al.*, 1992; De Hostos *et al.*, 1989). Although the related gene sequences show little homology within the 5' untranslated sequences or conservation in terms of overall intron/exon structure, there are several regions of the amino acid sequence which are strictly conserved. This observation implies that the related enzymes retain a limited number of sulphatase specific structural or catalytic motifs.

1.3.2 An Historic Overview

In 1907, Alois Alzheimer described the case of a 51 year old woman who was admitted to a hospital in Frankfurt because of memory impairment and what was described as *a strong feeling of jealousy toward her husband*. She became disorientated over time, incontinent, bedridden and died four and a half years after her admission (Amaducci *et al.*, 1991). A post-mortem examination of the patient, which was published by Perusini in 1910, showed evidence of demyelination of white matter in the central nervous system and also identified metachromatic deposits in the spinal cord when the tissue was stained with toluidine blue. This patient was thought to represent the first documented case of Alzheimer's disease, but a review of the clinical and histological findings suggest that the patient was actually the first to be described with the adult onset form of MLD (Amaducci *et al.*, 1991).

An earlier onset 'juvenile' form of MLD was subsequently described by Scholz in 1925 who also proposed that the disorder was caused by a myelin catabolic defect (reviewed by Kolodny and Fluharty, 1995). The most severe late-infantile form of MLD was first described by Greenfeld (1933) in two children who died at the age of three of what was then described as cerebral sclerosis. The clinical description of these two children was similar with birth and early development being normal in both cases. The first child walked at 11 months and began to speak at 16 months but walking difficulties developed at the age of 22 months and by 27 months could only stand when supported. The second child crawled but never walked without support because his legs crossed when walking was attempted. Tendon reflexes were absent in both children and they became bedridden before losing the ability to see or hear prior to death. Histological examination of the brain identified extensive demyelination of the white matter and the existence of lipoid containing granules in the nervous tissue (Greenfeld, 1933).

In 1955, von Hirsch and Peiffer were the first to correlate a diagnosis of MLD with the characteristic metachromasia observed in the cells of MLD patients (reviewed by Kolodny and Fluharty, 1995). An acetic acid-cresyl violet stain was found to differentiate lipid containing storage vesicles contained within cells of the nervous tissue of MLD patients. These stained storage vesicles gave rise to the distinctive metachromatic pattern of staining in the tissue. Austin (1959) and independently showed that this distinctive metachromasia was caused by an excess of stored cerebroside sulphate in the tissues of MLD patients. These metachromatic deposits were also found in presymptomatic individuals which demonstrated that they were not secondary to the breakdown myelin (Thomas *et al.*, 1977).

The most important biochemical milestone in the history of MLD was the identification by Austin (1963) of a deficiency of the enzyme arylsulphatase A (ASA) in brain, liver and kidney of two unrelated MLD patients as well as their affected sibs. A year later Mehl and Jatzkewitz (1965) demonstrated that ASA was involved in the degradation of cerebroside sulphate, a polar sphingolipid which is found predominantly as a component of myelin sheaths in the nervous system. In 1964 Mehl and Jatzkewitz also described a rare MLD patient whose clinical presentation was not caused by a deficiency of ASA but by the deficiency of a 'co-factor', which in normal individuals, acts to solubilise cerebroside sulphate and allows its subsequent hydrolysis (reviewed by Kolodny and Fluharty, 1995).

1.3.3 MLD Without ASA Deficiency

The degradation of sphingolipids relies on the presence of a series of non-enzymatic sphingolipid activator proteins (SAPs). Five sphingolipid activator proteins, the G_{M2} activator protein and four saposin homologues (A to D) which are produced from a single SAP-precursor have been identified (reviewed in O'Brien and Kishimoto, 1991; Fürst and

Sandhoff, 1992). Each of activator protein has a different lipid binding specificity (Klein *et al.*, 1994).

The degradation of cerebroside sulphate by ASA is catalysed by saposin B (SAP-1) which solubilises the lipid substrate to make it available to the ASA enzyme. SAP-1 was first described by Mehl and Jatzkewitz in 1964 (reviewed by Kolodny and Fluhart, 1995) after the identification of a non-enzymatic, heat stable protein which was necessary for the degradation of cerebroside sulphate by ASA (Li and Li, 1984). If the SAP-1 protein is absent, cerebroside sulphate cannot be solubilised or degraded and subsequently accumulates within the lysosome (Gieselmann *et al.*, 1991). The clinical presentation of patients with a SAP-1 deficiency therefore mirrors the presentation of patients with a deficiency of ASA, since both deficiencies cause the lysosomal storage of cerebroside sulphate.

Although SAP-1 deficient patients present with the clinical symptoms of MLD, they have normal levels of ASA activity in *in vitro* assays. SAP-1 is not required for ASA to degrade the artificial substrate, p-nitrocatechol, since the substrate is readily soluble in water. A SAP-1 deficiency will not therefore be detected by this assay (Henseler *et al.*, 1996). SAP-1 deficiencies can however, be differentiated by the assay of ASA activity in cultured skin fibroblasts using the natural ASA substrate, cerebroside sulphate. In the absence of exogenous SAP-1 or detergents which act to solubilise the substrate, SAP-1 deficient cells are unable to degrade cerebroside sulphate (Gieselmann *et al.*, 1994).

The SAP-precursor molecule is a 68 kDa glycoprotein which undergoes extensive proteolytic processing to produce the 12 kDa SAP-1 protein. Four SAP-1 mutations, two amino acid substitutions, T271I and C241S, an insertion, 777+1895C>A (reviewed by Gieselmann *et al.*, 1994) and a splicing defect (Henseler *et al.*, 1996). have been identified in seven patients presenting with 'atypical' MLD.

1.3.4 The Biochemical Diagnosis of MLD

Sulpholipid, is the general term used to categorise a variety of sulphur-containing lipids. Cerebroside sulphate is the most abundant sulpholipid substrate to be involved in the presentation of the lipidoses and is found in the highest concentration in the central and peripheral nervous systems (Kolodny and Fluharty, 1995).

ASA has been found to be deficient in all tissues from MLD patients which have been assayed, including skin, brain, kidney and liver (Jatzkewitz and Mehl, 1969). However, the biochemical diagnosis of MLD most commonly relies on the establishment of a deficiency of ASA in either cultured skin fibroblasts or peripheral blood leukocytes by the use of the artificial ASA substrate p-nitrocatechol sulphate or the natural substrate, cerebroside sulphate.

The assay of ASA with the water soluble artificial substrate, p-nitrocatechol sulphate, was originally described by Baum *et al.* (1959). Both ASA and the related sulphatase, ASB, show specificity for p-nitrocatechol. Various modifications to the original procedure of Baum have been reported to reduce the contribution of ASB to substrate degradation (Porter *et al.*, 1972; Lee-Vaupel and Conzelmann, 1987). The hydrolysis of p-nitrocatechol sulphate by ASA leads to the production of a colorimetric product which can be quantified.

ASA activity can also be measured using radiolabelled natural substrate and various authors have outlined methods for radioactively labelling cerebroside sulphate for this purpose (Poulos and Beckman, 1981). The assay of ASA activity using the natural substrate requires the presence of detergents or activator protein to solubilise the substrate. The hydrolysis of cerebroside sulphate can subsequently be measured by the release of radiolabelled sulphate. The hydrolysis of radiolabelled cerebroside sulphate can also be measured *in vivo*, by supplying cells in culture with the labelled substrate and quantifying the ability of the cells to internalise and degrade the exogenous substrate. This analysis is referred to as the cerebroside sulphate loading assay (Kihara *et al.*, 1980).

MLD patients excrete large amounts of cerebroside sulphate in urine and the measurement of this excess excretion has also been used to diagnose MLD (Philippart *et al.*, 1972). However, individuals with a deficiency of the activator protein, SAP-1, also excrete an excess of urinary cerebroside sulphate because of an inability to degrade the substrate. Therefore, although the measurement of excess urinary cerebroside sulphate is indicative of an inability to catabolise the substrate, it is not necessarily diagnostic of a deficiency of ASA.

1.3.5 The Clinical Diagnosis of MLD

MLD patients present with a broad spectrum of clinical phenotypes but the disorder is subdivided into three broad clinical subtypes which are based on the age of onset of clinical symptoms. Late-infantile patients present in their first years of life and do not usually live past the age of 10. Juvenile patients present between the ages of 3 years and puberty and adult patients present after puberty.(reviewed by Kolodny and Fluharty, 1995). All patients exhibit a deterioration of motor skills and other symptoms characteristic of a demyelinating disorder.

The clinical progression of MLD is characterised by a progressive loss of motor function which is caused by the breakdown of the myelin sheaths which surround nerves in both the peripheral and central nervous systems. The aberrant lysosomal storage of cerebroside sulphate within the nervous system causes extensive remodelling of the entire nerve fibre population. Nerve fibres from MLD patients therefore, tend to have abnormally thin myelin sheaths which causes a marked reduction in the speed at which nerves can conduct impulses (Thomas *et al.*, 1977; Bardosi *et al.* 1987).

The lysosomal storage of cerebroside sulphate, identified histologically as metachromatic deposits within nervous tissue, can be identified within all myelinating cells of patients with each of the MLD clinical subtypes (Bardosi *et al.*, 1987).

1.3.5.a Late-infantile MLD

Late-infantile MLD is the most common form of the disorder. In a review of 38 cases MacFaul (1982) found that these patients had an average age of onset of symptoms at 17 months, with most patients presenting with an immature gait or a deterioration in the walking ability if it had already developed. On average, normal children walk unaided by the age of 13 months, with 90% walking by 15 months. Fewer than 60% of late-infantile MLD patients walk before the age of 16 months and 15% never walk (Zlotogora *et al.*, 1981).

A loss of motor and cognitive skills (Sharpiro *et al.*, 1994) is illustrated by a deterioration of both social and language skills which are initially normal. By the age of three years most late-infantile MLD patients will have lost the ability to see (Goebel *et al.*, 1992) sit, speak or feed properly (MacFaul, 1982) and such patients rarely live past the age of ten.

1.3.5.b Juvenile MLD

The initial clinical course of both juvenile and adult MLD is characterised by symptoms which resemble those of schizophrenia or dementia or by difficulties at school or work. Behavioural problems of some description do generally tend to precede the onset of the neurological symptoms of MLD (Manowitz *et al.*, 1978). However, juvenile MLD can be subdivided into early and later onset forms on the basis of distinct clinical presentations. The early juvenile patients present between the ages of 4 and 6 years with motor difficulties such as a gait disturbance but with normal reflexes, *prior* to the onset of behavioural problems. In contrast, patients with the classical juvenile form of MLD present between the ages of 6 and 10 years with scholastic or behavioural problems well before the onset of neurological problems with balance and gait (Manowitz *et al.*, 1978).

1.3.5.c Adult MLD

Adult MLD is often difficult to diagnose because, like late-juvenile patients, individuals with adult MLD present with a range of psychological disorders, with neurological signs appearing later in the course of the disease (Baquis *et al.*, 1991; Reider-Grosswasser and Bornstein, 1987). Adult MLD patients do however, present with a distinct set of behavioural problems such as impulsiveness, poor judgement, emotional liability, social inappropriateness and poor attention span. These patterns of behaviour are consistent with symptoms which are commonly associated with dysfunctions of the frontal lobe and white matter disease, but are distinct from diseases such as Alzheimer's which affect cortical grey matter (Sharpiro *et al.*, 1994). Computerised axial topography (CAT) scans (Reider-Grosswasser and Bornstein, 1987) and magnetic resonance imaging (MRI) (Sharpiro *et al.*, 1994) have been used to identify severe demyelination within frontal areas of the brain from adult-MLD patients.

The presentation of the later onset MLD patients with behavioural and scholastic difficulties is perhaps best summarised by a clinical review presented by Manowitz *et al.* (1978).

Patient RK had a normal pre and postnatal history and did well academically until the 10th grade when there was an abrupt decline in her grades. She eventually barely graduated from her high school in the bottom 10 % of her class. She was hospitalised at the age of 18 years and her admission report read, *On admission she was mildly elated in a silly way, she was smiling and superficially cheerful which was not at all in keeping with her situation. She was ambivalent and inconsistent toward her mother, perplexed, helpless and gave up easily. Her vocabulary was good, she was correctly orientated, her memories were only fair with poor memory retention and recall poor. Her attention was easily gained but the span was extremely short.* At the age of 22 she could not walk without assistance and was incontinent

of urine and faeces. A sural nerve biopsy examination revealed a disruption of the myelin sheath. At 27 years of she was generally weak in the arms and legs and had dilated pupils which did not respond to light.

Although a deficiency of ASA leads to an accumulation of cerebroside sulphate in the nervous system and the pathogenic development of MLD, the substrate also accumulates in the kidneys, liver, spleen, lymph nodes, testis, pituitary, bone marrow and gall bladder without generally affecting the functions of these organs (Siegel *et al.*, 1992). However, Siegel *et al.* (1992) described the presentation of a 27 year old adult-MLD patient with severe gastrointestinal (GI) bleeding. The storage of cerebroside sulphate in the gall bladder was hypothesised to cause the development of neoplastic growths in the organ and subsequently GI bleeding. This hypothesis was supported by similar observations made by Warfel *et al.* (1984).

Baquis *et al.* (1991) described a 30 year old adult-MLD patient who was periodically incontinent, had poor speech, was easily distractible and showed impulsive behaviour. His deep tendon reflexes were absent, there was a slowing of nerve conduction speed and the presence of 'granular' material in nervous tissue. Earlier, at the age of six years the patient had presented with a bilateral pes cavus foot deformity, which preceded the onset of behavioural difficulties by many years. This early presentation of pes cavus foot deformity gives an indication of the 'lifelong' nature of the disorder with peripheral nervous system signs emerging prior to central nervous system abnormalities.

1.3.6 The Molecular Genetics of MLD

The 1.6 kb ASA cDNA was isolated in 1989 by Stein *et al.* (1989) and was found to encode a 62 kDa polypeptide of 507 amino acids in length. The amino acid sequence has three potential N-glycosylation sites, all of which are used. The genomic copy of the gene

was identified on the long arm of human chromosome 22 at 22q13.3 (Woollart *et al.* 1996) and has been cloned (Kreysing *et al.*, 1991). The gene was found to span only 3.2 kb of genomic DNA and has eight small exons and seven introns (Figure 1.2) (reviewed by Gieselmann *et al.*, 1994). No regulatory elements were identified in the 5' untranslated region of the gene beside a CpG island which is a characteristic of house keeping genes (Kreysing *et al.*, 1991). The gene produces three different mRNA species of 2.1, 3.7 and 4.8 kb in length. Each of the mRNAs produce an identical polypeptide and therefore the mRNAs differ only in the amount of 3' untranslated sequence that they contain (Gieselmann *et al.*, 1989).

Two common mutations, which each account for approximately 25% of MLD alleles, have been identified within the ASA gene. These mutations can also account for the presentation of the three clinical subtypes of MLD (Polten *et al.*, 1991). Homozygosity for one of these mutations, which alters the first base of intron 2 (459+1 G>A) and affects a splice donor site, correlates with the presentation of the most severe, late-infantile form of MLD. Alleles containing this mutation produce no functional ASA protein because of the effect of the mutation on splicing (Polten *et al.*, 1991). The other common mutation is a C to T transition at nucleotide number 1277 of the ASA cDNA which causes the substitution of a leucine residue for a proline (P426L) (Figure 1.2). Individuals who are homozygous for the P426L mutation develop the adult form of MLD. An ASA allele which contains the P426L mutation produces ASA in normal amounts and the enzyme retains catalytic activity, but it has a drastically reduced half life. It is the increase in the rate of degradation of the enzyme which is responsible for the deficiency of ASA in P426L homozygotes (Polten *et al.*, 1991). Compound heterozygotes for the late-infantile mutation, 459+1G>A and the adult mutation, P426L, present with the intermediate juvenile form of the disease (Polten *et al.*, 1991).

Prior to the commencement of this project, only eight pathogenic MLD mutations were identified as well as three sequence variants which did not cause disease. A total of 28

ASA mutations have now been reported. These mutations have been summarised by Gieselmann *et al.* (1994) and are presented in Figure 1.2. Generally, there seems to be good correlation between the degree of deficiency of ASA and the severity of disease. Mutations which produce no ASA activity cause late-infantile MLD, those which produce some residual ASA activity are responsible for the development of adult MLD and compound heterozygotes for late-infantile and adult alleles present with juvenile MLD.

1.3.7 ASA Pseudodeficiency

Following the initial finding of Austin *et al.* (1963) that it was a deficiency of the lysosomal enzyme arylsulphatase A that was responsible for the clinical presentation of MLD, it became increasingly apparent that not all “deficiencies” of ASA lead to the presentation of the disease. Dubois *et al.* (1975) was the first to report such a case, in which an obligate MLD heterozygote had a level of ASA activity well below that expected of an asymptomatic carrier. This observation was reinforced by that of Lott *et al.* (1976) who described a similar case where, in two MLD families, one apparently healthy parent *had enzyme values in the homozygous range of MLD* such that standard enzymatic assays (Baum *et al.*, 1959) could not differentiate between the pathogenic and non-pathogenic classes of ASA deficiency. Clinically normal individuals were said to be ‘pseudodeficient’ for ASA if their enzyme deficiency is difficult to distinguishable from those of MLD patients (Wenger and Louie, 1991).

The obvious problem posed by the identification of pseudodeficient individuals was in regard to the prenatal diagnosis of MLD. A measured deficiency of the ASA enzyme in

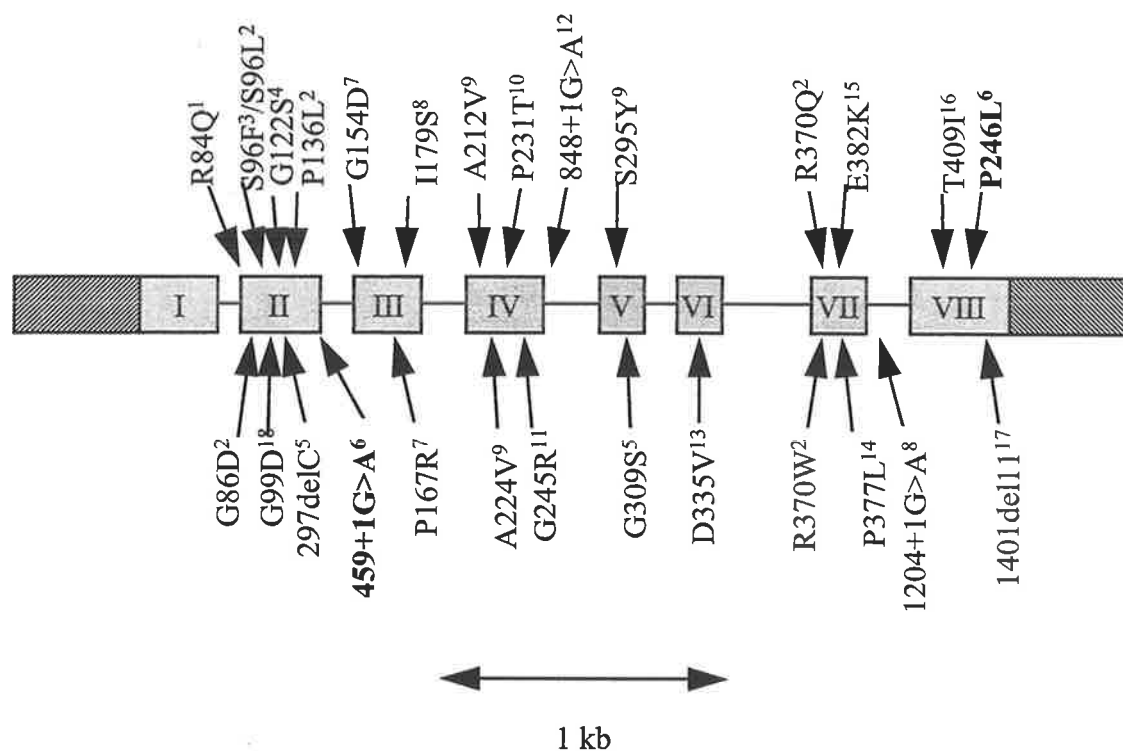


Figure 1.2 Structure of the ASA gene and disease-causing mutations

The eight exons of the ASA gene are shaded and numbered and the 5' and 3' untranslated regions are hatched. The position of the 28 known MLD mutations are indicated on the figure and referenced below. Single letters correspond to the amino acid letter code and numbers to the amino acid number within the ASA polypeptide which is altered. The two most common mutations are in bold. The scale bar indicates the proportions of the gene and of the intron/exon sequence. ¹Kappler *et al.* (1992); ²Heinisch *et al.* (1995); ³Gieselmann *et al.* (1991); ⁴Honke *et al.* (1993); ⁵Kreysing *et al.* (1993); ⁶Polten *et al.* (1991); ⁷Kappler *et al.* (1994); ⁸Fluharty *et al.* (1991); ⁹Barth *et al.* (1993); ¹⁰Caillaud *et al.* (1993); ¹¹Hasegawa *et al.* (1990); ¹²Pastor-Soler *et al.* (1993); ¹³Gieselmann *et al.* (1994); ¹⁴Zlotogora *et al.* (1994); ¹⁵Barth *et al.* (1994); ¹⁶Hasegawa *et al.* (1994); ¹⁷Bohne *et al.* (1991); ¹⁸Kondo *et al.* (1991).

prenatal samples no longer necessarily meant that the pregnancy would result in a child with MLD. Dubois *et al.* (1977), recognised the gravity of this diagnostic dilemma, particularly on occasions when pseudodeficient parents had MLD affected children and advised caution in the use of prenatal ASA measurements in diagnosing MLD in such families. Further diagnostic difficulties were presented by Butterworth *et al.* (1978) when documenting the case of a child with a degenerative neurological disorder who also had low ASA activity. The child's father was found to be pseudodeficient for ASA. This report illustrated that not all cases of low ASA activity and neurodegeneration should be diagnosed as MLD because the clinical presentation of the child was subsequently shown to be unrelated to low ASA activity.

Wenger and Louie (1991) generally defined enzymatic pseudodeficiencies as *the in vitro measurement of low activity of an enzyme in a healthy person such that pseudodeficient individuals may be hard to distinguish from presymptomatic adult onset patients.*

1.3.7.a Biochemical analysis of ASA pseudodeficiency

Two different deficiencies of ASA have been identified and each has a distinct clinical outcome. The first results in the development of the 'classical' clinical symptoms of MLD whilst the second is associated with an asymptomatic pseudodeficiency of the ASA enzyme. Chang and Davidson (1983) and Herz and Bach (1984) independently demonstrated that the same enzyme was involved in the manifestation of both deficiencies. Both groups showed that the enzymes involved had identical substrate affinity, optimal pH and sensitivity to heat denaturation. Importantly, Chang and Davidson (1983) revealed that the two enzymes had slightly different structures which was demonstrated by their different isoelectric points. Herz and Bach (1984) concluded that ASA pseudodeficiency was simply due to the ASA enzyme being present in lower amounts and estimated that this reduction was of the order of 85%.

Kihara *et al.* (1980) used the cerebroside sulphate loading assay to resolve the problem of differentiation between MLD patients and individuals who were pseudodeficient for ASA. Cultured cells from individuals who do not have MLD, are able to hydrolyse intracellularly incorporated cerebroside sulphate whereas cells cultured from an infantile MLD patient lack this ability. The rate of hydrolysis measured in cells cultured from a pseudodeficient individual does not differ from that of control cells. The assay was used to prenatally diagnose a case of MLD where the parents were pseudodeficient for ASA and standard assays (Baum *et al.*, 1959) were unable to differentiate between the parents and propositus. After studying the measured levels of ASA in the family, the authors hypothesised that one of the parents was probably a compound heterozygote for an MLD mutation and a pseudodeficiency variant and that this genotype accounted for the difficulty in unequivocal diagnosis (Kihara *et al.*, 1980).

1.3.7.b Molecular analysis of the ASA pseudodeficiency allele

Langenbeck *et al.* (1977) hypothesised that a pseudodeficiency of ASA was caused by an asymptomatic sequence variant within the ASA gene and that the different deficiencies of the enzyme were simply due to an allelic series of ASA mutations. In Langenbeck's model for the inheritance of MLD and ASA-PD he designated the normal ASA allele A, the classical deficiency allele A' and one which encoded diminished yet asymptomatic levels of activity A". The AA' heterozygous genotype accounted for the 50% reduction in activity measured in heterozygotes and the A"A" genotype resulted in the asymptomatic reduction in ASA activity observed in pseudodeficient individuals. In MLD families where one parent had very low, albeit asymptomatic levels of ASA activity, Langenbeck *et al* (1977) proposed that such parents could be compound heterozygotes for a classical MLD mutation (A') and a pseudodeficiency allele (A") which encoded diminished ASA activity.

With the isolation of the ASA gene, the molecular defect responsible for ASA pseudodeficiency was identified by Gieselmann *et al.* (1989). It was recognised that the ASA gene produced three different mRNA species of 2.1, 3.7 and 4.8 kb in length, which varied only in the amount of 3' untranslated sequence that they contained (Figure 1.3) as all mRNA species encoded an identical 507 amino acid ASA polypeptide.

Adhering to the original model proposed by Langenbeck *et al.* (1977), the pathogenic and non-pathogenic deficiencies of ASA were indeed identified as allelic variants of the same gene. The ASA gene sequence of normal and pseudodeficient individuals were in fact differentiated by two A to G changes, the first of which caused the substitution of a serine residue for a glycosylated asparagine at amino acid number 350 (N350S) of the ASA polypeptide (Figure 1.3). The loss of the glycosylated residue accounted for the observed structural differences in size and isoelectric point between the wild type ASA and ASA-PD proteins (Fluharty *et al.*, 1983; Chang and Davidson, 1983). When N350S was introduced into the normal ASA cDNA and transiently expression in COS cells, it was reported to have no effect on the activity of the ASA enzyme in terms of rate of synthesis, stability or substrate affinity (Gieselmann *et al.*, 1989).

The reduction in ASA activity seen in pseudodeficient individuals was however, attributed by Gieselmann *et al.* (1989) to the second A to G change which altered the sequence of the major 2.1 kb polyadenylation signal AATAAC to AGTAAC. Northern analysis showed that the 2.1 kb mRNA species accounted for an estimated 90% of the total ASA mRNA in normal individuals and that this transcript was absent in individuals homozygous for the ASA-PD allele (Figure 1.3). The deficiency of this transcript provided an explanation for what Gieselmann *et al.* (1989) estimated as the 10% residual ASA activity seen in individuals homozygous for the ASA-PD allele. This finding also accounted for previous observations (Chang and Davidson, 1983; Herz and Bach, 1984) concerning a

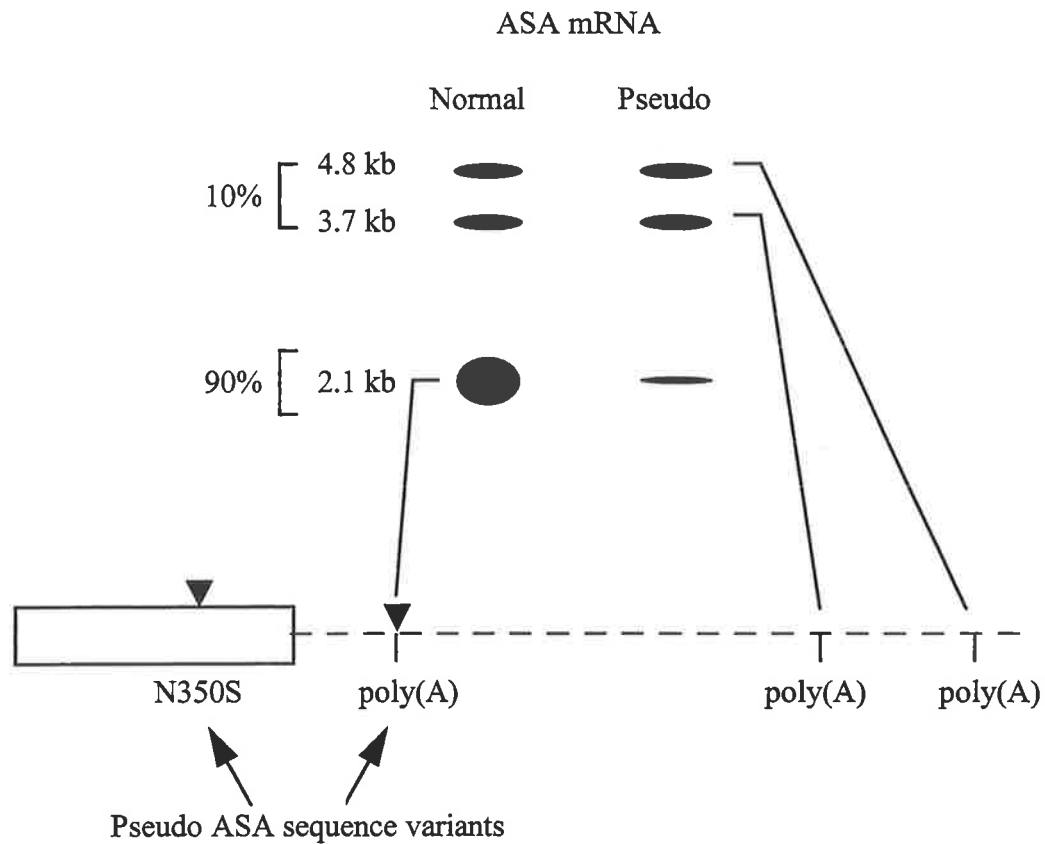


Figure 1.3 The arylsulphatase A pseudodeficiency allele

The boxed area represents the ASA coding sequence and the broken line represents the 3' untranslated region of the ASA gene. The three ASA polyadenylation sites are marked (poly A). The sequence variants which constitute the ASA pseudodeficiency allele are labelled and their relative positions in the ASA gene are marked with small arrows. The reported frequency of each ASA mRNA species is represented for normal and pseudodeficient individuals.

proposed reduction in the *amount* of ASA protein as opposed to a reduction in the *activity* of the ASA enzyme being responsible for ASA pseudodeficiency.

The carrier frequency of the ASA-PD allele has been estimated in a variety of populations. Nelson *et al.* (1991), Chabás *et al.* (1993) and Barth *et al.* (1994) identified the ASA-PD allele at a frequency of 12.3%, 9.4% and 12.7% of the Australian, Spanish and British populations respectively. Nelson *et al.* (1991) and Barth *et al.* (1994) also recognised that although N350S was always inherited in combination with the polyadenylation defect, it could also be inherited independently, with N350S being found alone with a frequency of 4.8% and 5.2% of the Australian and British populations respectively. Zlotogora *et al.* (1994) proposed a founder effect to explain the co-inheritance of the two alterations and suggested that N350S probably occurred first, thus accounting for its inheritance without the polyadenylation defect.

The conclusion that N350S had no effect on the activity of the ASA enzyme has been questioned. Francis *et al.* (1993) described a pedigree where individuals had various combinations of normal, MLD and pseudodeficiency alleles and importantly, identified an individual who carried the N350S allele without the polyadenylation defect. This individual was also an obligate MLD heterozygote (N350S/ASA⁻). The measured level of ASA activity in this individual was significantly below that expected of a normal MLD heterozygote suggesting that N350S played a role in defining the biochemical pseudodeficiency phenotype. Similarly, Shen *et al.* (1993) identified a family where a father and two children were compound heterozygotes for an MLD-causing allele and an allele containing N350S without the polyadenylation defect. Again the level of ASA measured in these individuals was less than that expected for obligate carriers of MLD. The N350S allele was estimated by Shen *et al.* (1993) to reduce ASA activity to 12%-21% of normal. If this is indeed the case then N350S would significantly contribute to the reduction in the level of ASA expression

observed in pseudodeficient individuals carrying both the polyadenylation and N350S defects.

1.3.7.c Neurodegeneration and ASA-pseudodeficiency

The question of whether a pseudodeficiency of ASA is involved in the presentation of MLD or any other demyelinating disorder has been widely debated particularly in cases where individuals were compound heterozygotes for the ASA-PD allele and a null MLD mutation. After identifying the molecular defects associated with ASA-PD, Gieselmann *et al.* (1989) posed the question of whether such individuals would develop neurological symptoms later in life, in his concluding remarks. The question was addressed by Penzein *et al.* (1993) who studied 16 such compound heterozygotes and found no evidence to suggest that this combination of alleles was involved in the presentation of MLD. Although two of the compound heterozygous individuals showed neurological symptoms, they were non-progressive and did not resemble symptoms associated with MLD. It was concluded that their symptoms were unrelated to the reduction in ASA activity. This finding was confirmed by Francis *et al.* (1993) who found no evidence of the clinical manifestation of MLD in a similar group of compound heterozygotes.

1.3.7.d ASA pseudodeficiency and other demyelinating disorders

A link between compound heterozygosity for the ASA-PD and MLD alleles and the development of 'atypical MLD' or other demyelinating disorders such as multiple sclerosis (MS) have been widely speculated. A population carrier frequency of the ASA-PD allele of approximately 1 in 5 ensures that carriers of a variety of neurodegenerative disorders will also by chance carry the ASA-PD allele. The screening of one hundred and sixty MS patients for

the presence of the ASA-PD allele found no significant difference in the frequency of the allele when compared to a control population of equal size, although ASA-PD homozygotes were identified at a frequency of 2.5% as opposed to the control population frequency of 0.6% (Kappler *et al.*, 1991a).

ASA activity was also measured in 140 patients with epilepsy or mental retardation to determine whether the presence of the ASA-PD allele was in any way linked to their presentation (Sangiorgi *et al.*, 1991). None of the individuals showed any evidence of urinary cerebroside sulphate excretion which, as described in 1.3.4, is diagnostic of the *in vivo* accumulation and storage of cerebroside sulphate due to a deficiency of ASA function (Sangiorgi *et al.*, 1991). The patients were not genotyped for the ASA-PD allele but from enzymatic data, the authors suggested that there was a disproportionate increase in the number of ASA-PD carriers and homozygotes. Although there seems to be no direct evidence to link the presentation of these patients with the presence of the ASA-PD allele, it has been suggested that a lowering of ASA levels may somehow predispose the nervous system to degradation or dysfunction *via* other multifactorial environmental or genetic factors.

The notion that low ASA activities may be involved in the atypical presentation of other neurological disorders is a theme touched on by numerous of other authors. Weinberger *et al.* (1994) presented the case of a ASA-PD homozygote who began to show the neurodegenerative signs of adult MLD after a suicide attempt led to severe oxygen deprivation. Demyelination is an uncommon complication of carbon monoxide poisoning or any other sort of severe hypoxia and in this case it was postulated that the greatly increased need for myelin turnover following hypoxia exceeded the patients residual capacity of ASA to manage myelin catabolism, thus causing the accumulation of cerebroside sulphate and the onset of MLD like symptoms. Tinuper *et al.* (1994) offered similar arguments for the presentation of an ASA-PD homozygote with an atypical form of Lafora disease, a

neurodegenerative disorder characterised by epilepsy and progressive dementia. The author by no means exclude the possibility of fortuitous association, but the paper again raised the question of whether low ASA activity may play a role in the atypical presentation of other neurodegenerative disorders.

In a converse argument Kappler *et al.* (1991b) presented the case of a family of three children with low ASA activities whose presentation with 'atypical MLD' had nothing to do with their pseudodeficient levels of ASA activity. The patients showed no evidence of mental retardation, slowing of nerve conduction or dementia and excreted no urinary cerebroside sulphate. This observation, again serves as a diagnostic warning that not all patients with low ASA activity and degenerative neurological disease have MLD and that this may account for the large numbers of reports of patients with 'atypical ' forms of the disorder.

1.4 GENOTYPE TO PHENOTYPE CORRELATIONS

As mentioned previously, patients with MLD and indeed patients with numerous other lysosomal storage disorders, present with a broad spectrum of clinical phenotypes (reviewed by Scriver *et al.*, 1995). It has been proposed by numerous authors that this variation in clinical presentation is due to the existence of an allelic series of mutations at each disease locus which encode variable levels of residual enzyme activity (McKusick *et al.*, 1972; Neufeld and Muenzer, 1989).

Generally, within the lysosomal storage disorders, there seems to be a good correlation between residual enzyme activity and the severity of disease. The greater the deficiency of an enzyme the more severe the disease with which a patient will present conversely, disease progression tends to be modified by an allele which expresses some residual enzyme activity. The level of enzyme activity needed to offer some "protection" against the development of

severe disease may be only a fraction of the normal activity of the enzyme (Neufeld, 1991). In the case of MLD, homozygosity for severe ASA mutation lead to the development of the late-infantile form of the disorder however, ASA mutations which produce some residual ASA activity modify this severe presentation and result in the development of juvenile or adult MLD.

The lysosomal storage disorder, Tay-Sachs disease, is common among the Ashkenazi Jews and is caused by mutations in the α -subunit of the β -hexosaminidase gene (reviewed by Gravel *et al.*, 1995). A single severe frame shift mutation which causes the premature termination of translation accounts for 70% of mutant alleles among the Ashkenazi Jews and is responsible for the development of the most severe form of Tay-Sachs disease (Myerowitz and Costigan, 1988). A second Tay-Sachs mutation G269S, produces some residual enzyme activity, which modifies the severe presentation of the disease (Navon and Proia, 1989). Tay-Sachs disease therefore provides a second example of a disorder in which there is a strong correlation between genotype and clinical presentation.

Conzelmann and Sandhoff (1983/1984) put forward a kinetic model to explain the presentation and variation in clinical onset of the lysosomal storage disorders in terms of a “critical threshold of enzyme activity”. This level of activity was proposed to be sufficient to degrade all substrate entering the lysosome. If however, the level of residual enzyme activity fell below this threshold it would result in the accumulation of substrate within the lysosome and the onset of disease. The severity of disease was proposed in this model to be governed by the extent to which an enzyme was deficient which in turn governed the rate of substrate accumulation (Conzelmann and Sandhoff, 1983/1984). This model suggested that small alterations in the activity of a lysosomal enzyme could have a great effect on disease presentation by varying the rate of substrate accumulation.

The relationship between genotype and phenotype can however be complex. In the case of MLD, Kreysing *et al.*, (1993) reported the identification of a patient with late-infantile MLD who had a *high* level of residual ASA activity. The child was shown to be a compound heterozygote for the severe MLD mutation 459+1G>A and a novel G309S mutation. Expression of the G309S mutation showed that it encoded a level of ASA that was comparable to that measured in ASA-PD homozygotes (about 10% of normal). The presentation of this patient with late-infantile MLD was hypothesised to be caused by the instability of ASA containing G309S in oligodendrocytes, causing its rapid degradation before reaching the lysosome.

Another example of a poor correlation between genotype and clinical phenotype is presented by the related X-linked peroxisomal disorders, adrenoleukodystrophy and adrenomyeloneuropathy (reviewed by Moser *et al.*, 1995). Although these disorders are clinical variants of the same disease, they have repeatedly been found to occur within the same family, despite the presence of a single disease causing mutation. This variation in clinical presentation is hypothesised to be caused by the presence of a modifier gene (Davis *et al.*, 1979).

One of the major objectives of the identification of disease causing mutations is to enable the prediction of the severity of disease with which patients will present. The large number of mutations that have been identified as causing the respective lysosomal storage disorders (reviewed by Scriver *et al.*, 1995), means that most patients will present with disease because of the combination of two different disease causing alleles. Given that there are many ways in which to prevent the formation of functional enzyme, non-functional alleles are expected to be common and the clinical phenotype which results from the inheritance of these different non-functional alleles is expected to be similar. However, the number of mutations which produce some residual enzyme activity and result in the development of

milder disease, are expected to be limited in number (Neufeld, 1991). Alleles which express some residual enzyme activity may be susceptible to modification by factors such as modifier genes which would account for observed differences in the presentation of mild patients with similar genotypes, since small alterations in the residual activity of an enzyme may have a great effect on the presentation of disease (Conzelmann and Sandhoff, 1983/84). This serves as a point of caution when correlating genotype and phenotype, even in the case of MLD where this correlation is generally sound.

1.5 THE TREATMENT OF LYSOSOMAL STORAGE DISORDERS

There is currently no known treatment for MLD that will halt the progressive nature of the disorder. Initial attempts at treatment were based on alterations to the dietary intake of sulphur (Moser *et al.*, 1967) and vitamin A (Warner, 1975). Since, both sulphur and vitamin A are required for the synthesis of cerebroside sulphate, it was reasoned that there reduced intake would slow the synthesis and subsequent lysosomal accumulation of cerebroside sulphate within the nervous system.

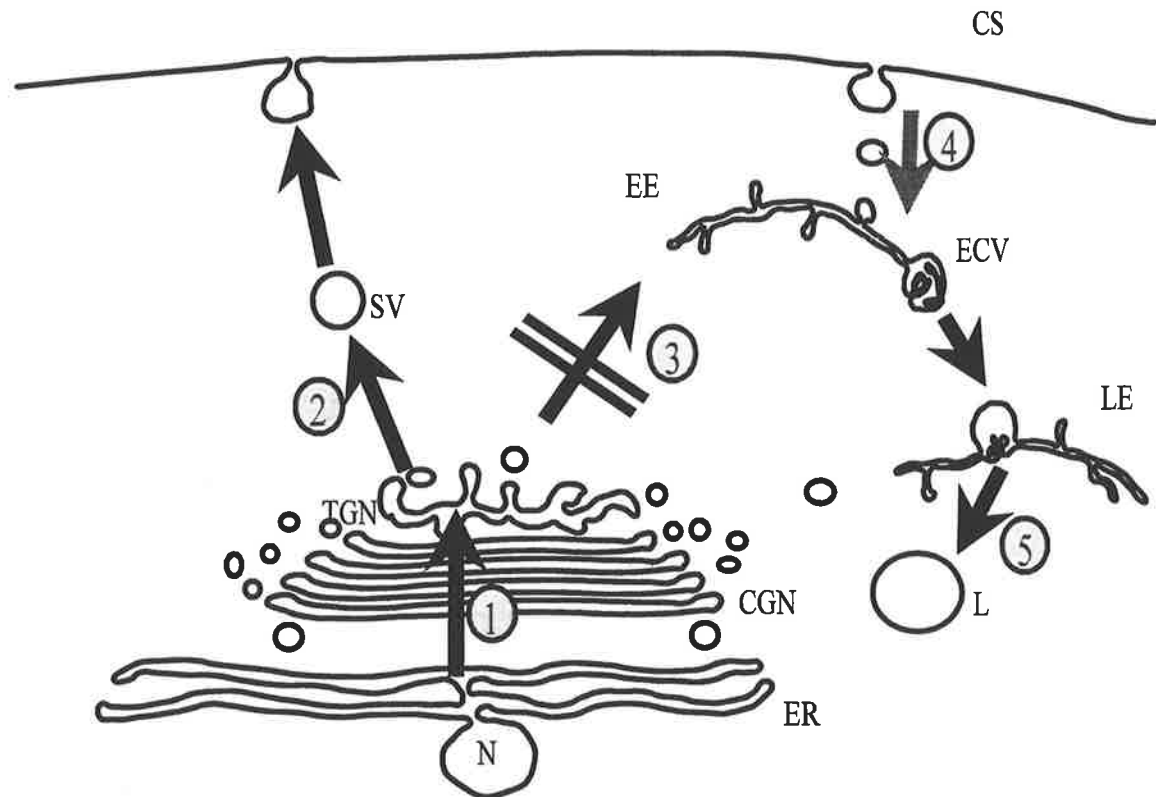
The role of the mannose 6-phosphate receptors in the targeting of enzymes to the lysosome has been well established. Mannose 6-phosphate receptors are however, also present on the cell surface, where they are involved in the internalisation of a proportion of the synthesised lysosomal enzyme that is secreted from the cell (Chao *et al.*, 1990), into the pre-lysosomal compartments (refer to Figure 1.1) (Matovcik *et al.*, 1990).

Neufeld *et al.* (1991) demonstrated that the storage of substrate in cultured cells from patients with a lysosomal storage disorder could be corrected by the co-culture of the effected cells with normal cells. This correction was subsequently shown to be caused by the internalisation, by the lysosomal storage disorder cells, of the deficient enzyme which had

been excreted into the media by the normal cells in culture (reviewed in Bou-Gharios *et al.*, 1993). This correction of storage has been demonstrated for cells deficient for ASA (Wiesmann *et al.*, 1971).

The internalisation of exogenous lysosomal enzyme by membrane bound mannose 6-phosphate receptors is the basis of therapies designed to replace deficient enzymes in lysosomal storage disorder cells *in vivo* (Figure 1.4). However, although the supply of exogenous enzyme has successfully been used to treat Gaucher disease, a lysosomal storage disorder, it has never been successful in the treatment of a disorder that has central nervous system pathology, such as MLD. Several attempts have however been made to directly administer preparation of arylsulphatase A to MLD patients either by injection of enzyme directly into the blood stream or by the implantation of human amnion, which is non-antigenic and rich in lysosomes, into MLD patients. Neither of these treatments were found to reverse the storage of cerebroside sulphate within the neural tissue of MLD patients.

Gaucher disease is a common lysosomal storage disorder among the Ashkenazi-Jewish population and is caused by a deficiency of β -glucosidase. A deficiency of β -glucosidase causes the accumulation of glucosylceramide. Although there are three distinct clinical subtypes, by far the most common form of the disorder is type I Gaucher disease, which is characterised by a dysfunction of macrophages which causes enlargement of the spleen and liver and skeletal abnormalities. There is however, no central nervous system involvement in type I Gaucher disease (reviewed in Beutler *et al.*, 1995). Gaucher disease is an example of a lysosomal storage disorder which has been successfully treated by enzyme replacement therapy, by the direct injection and targeting of β -glucosidase to the affected macrophages. Bone marrow transplantation (BMT) has also been used to successfully treat patients with Gaucher's disease.



adapted from Hopwood and Brooks (1996)

Figure 1.4 Treatment of lysosomal storage disorders by enzyme replacement therapy

The uptake of exogenous lysosomal enzyme by storage disorder cells via cell surface mannose 6-phosphate receptors and there transport to the early endosome. CS: cell surface; EE: early endosome; LE: late endosome; L: lysosome; N: nucleus; EE: early endosome; ECV: endocytic vesicle; SV: secretory vesicle; TNG: trans-Golgi network; CNG: cis-Golgi network.

- 1) Transportation of proteins from the ER to the CGN via membrane bound vesicles.
- 2) Secretion of proteins via the secretory pathway.
- 3) Enzymatic block in the synthesis of lysosomal enzymes.
- 4) Internalisation of exogenous lysosomal enzymes.
- 5) Maturation of late-endosomes into mature lysosomes containing exogenously supplied lysosomal enzyme.

The rationale behind treating patients with lysosomal storage disorders by BMT, stems from the observation that the metabolic defect in storage disorder cells can be corrected by their co-culture with normal macrophages or lymphocytes. This suggests that normal circulating macrophages and lymphocytes (which would be produced by the transplanted marrow) should have the ability to deliver normal enzyme to effected tissue in lysosomal storage disorder patients and reverse the storage of substrate within these tissues. In addition, macrophages have the ability to cross the blood/brain barrier and transform into microglial cells. These cells should hypothetically be a source of exogenous enzyme within the central nervous system.

More than twenty MLD patients have now been treated by BMT. Although the effect of this treatment on the progression of disease have been variable, in general the treatment has raised *in vivo* levels of ASA and slowed the progression of disease (reviewed by Kolodny and Fluhrt, 1989). This has been well illustrated by the comparison of treated and untreated children in a single family, where the progression of disease and the onset of symptoms has been modified in the treated children (Krivit *et al.*, 1990). Although BMT has been successful in slowing the progression of MLD, it has not halted the progression of the disease. The progression of disease in BMT treated MLD patients was also observed to continue and even accelerate for up to a year after transplantation, before a slowing in the progression of the disorder was observed. Krivit *et al.* (1992) subsequently recommended against the that BMT in patient in which the clinical symptoms of MLD were rapidly progressing and that only presymptomatic individuals were likely to benefit from the treatment.

1.6 AIMS OF THE PROJECT

The broad objectives of this project are to investigate the development of MLD in the patient population referred to the Women's and Children's Hospital to gain further insight

into the molecular and biochemical defects which cause disease. This analysis will add to our understanding of the structure and function of ASA and of the related sulphatase enzymes. The specific aims of this thesis are;

1) To develop methods for the detection of known MLD mutations and subsequently, novel sequence alterations within the ASA gene and to use these protocols to identify the molecular defects that are responsible for the clinical presentation of MLD;

2) To generate an understanding of the mechanisms underlying the variation in severity seen in MLD patients by correlating patient genotype with clinical phenotype;

3) To re-evaluate the ASA-PD allele and to quantify the effect that it has on the activity of the ASA enzyme in view of the role that I hypothesise it may play in the development of disease;

4) To add to our understanding of the function of the ASA protein and to determine the biochemical phenotype of each patient allele by developing a system of assay which will allow the quantification of the effect that different sequence alterations within the ASA gene have on the activity of the ASA enzyme.

The significance of this project lies in the need to resolve the confusion caused by the existence of “pseudodeficiencies” of ASA activity that can be misinterpreted as levels of activity that may cause MLD. I hypothesise that alleles which cause pseudodeficiencies of ASA may play a role in modifying the course of MLD or may be responsible for the development of disease. This study is therefore fundamentally important in determining the role that pseudodeficiency alleles play in the development of MLD and of other related disorders.

Chapter 2

Materials and Methods

2.1 MATERIALS

2.1.1 Clinical Presentation of MLD Patients

Patient	Age at Diagnosis	Clinical Presentation	MLD subtype
MM	8 yrs	At diagnosis, demented, signs of neurodegeneration, abnormal nerve conduction, CT scan revealed white matter degeneration	Juvenile
WT	2 yrs	Began deteriorating at age 2 yrs, axial hypotonia, floppy neck. Gradual lessening of responsiveness, fits, peripheral neuropathy.	Late-infantile
LE	18 months	Wide based gait, weight on toes, knee jerks reduced, ankle jerks absent. CT scan showed widespread neuropathy, slowing of nerve conduction.	Late-infantile
LP	5 yrs	Tremors, signs of peripheral neuropathy and pes cavus at diagnosis, raised CSF protein, slow nerve conduction. Could read well at five yrs, by 11 years had regressed to 3-4 yo, incontinent.	Juvenile
SA	2 yrs	From age 2 yrs, refused to walk, weakness in the legs, abnormal nerve conduction, died at 5 yrs.	Late-infantile
PG	5 yrs, 6 months	At diagnosis showed motor problems, difficulty in standing, unable to hold a pen, little spontaneous speech at 7 yrs, ataxic, extended plantars, muscle wasting.	Juvenile

2.1.2 Electrophoresis

Reagents for electrophoresis were obtained from:

Agarose, type 1	Progen Industries, Darra, QLD, Australia
Ammonium persulphate	Ajax Chemicals Pty Ltd, Auburn, NSW, Australia
Bromophenol blue	BDH Chemicals Ltd, Poole, Dorset, UK

Ethidium bromide	Boehringer Mannheim, Germany
Formamide	Ajax Chemicals Pty Ltd, Auburn, NSW, Australia
Molecular weight markers SPPI/ <i>EcoRI</i> and pUC19/ <i>HpaII</i>	Bresatec, Adelaide, SA, Australia
N,N,N',N'-tetramethyl- ethylenediamine (TEMED)	Bio-Rad Laboratories, Hercules, CA, USA
Sequagel sequencing system	National Diagnostics, Atlanta, Georgia, USA
Urea	Ajax Chemicals Pty Ltd, Auburn, NSW, Australia
Xylene cyanol	Tokyo Kasei, Tokyo, Japan

2.1.3 Enzymes

All restriction endonucleases used in this study were obtained from Boehringer Mannheim unless otherwise stated. Other enzymes were obtained from the following suppliers:

Alkaline phosphatase (from calf intestine)	Boehringer Mannheim, Germany
Proteinase K	Boehringer Mannheim, Germany or Amersham, UK
RNasin [®]	Promega Corp, Madison, WI, USA
T4 DNA ligase	Promega Corp, Madison, WI, USA; Boehringer Mannheim, Germany
T4 polynucleotide kinase	Pharmacia, Uppsala, Sweden
<i>Taq</i> DNA polymerase	Perkin Elmer Cetus, Norwalk, CT, USA; Biotech International Ltd, Perth, Australia

2.1.4 Radiochemicals

[γ - ³² P] ATP (6000Ci/mmol)	NEN [®] DuPont, Boston, MA, USA
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2.1.5 Antibiotics

Ampicillin	Sigma Chemical Co, St Louis, MO, USA
Penicillin/Streptomycin	CSL Ltd, Parkville, VIC, Australia

2.1.6 Bacterial Strains

The *E. coli* strains used in this study are described below:

DH5 α F'	<i>supE44, hsdR17, (r_k⁻, m_k⁺), thi-1, recA1, endA1, gyrA, (Nal^r), relA1, ∇(lacZYA-argF)U169, deoR, F', (Φ80lacZ∇M15) (Hanahan <i>et al.</i>, 1993)</i>
MC 1061	<i>F⁻, araD139, ∇(ara-leu)7696, galE15, galK16, ∇(lac)X74, rpsL (Str^r), hsdR2, (r_k⁻ m_k⁺), mcrA, mcrB1 (Wertman <i>et al.</i>, 1986)</i>

2.1.7 Vectors

All vectors which were used in this study are listed below:

2.1.7.a Plasmid vectors

pGEM [®] -T System 1 PCR cloning vector	Promega Corp, Madison, WI, USA
pRSVN.SV40 expression vector	Obtained from Dr Alan Robins, Department of Biochemistry, University of Adelaide, Adelaide, Australia
HT14/CP8 ASA cDNA	Supplied by Dr Volkmar Gieselmann, Christian Albrechts University, Kiel, Germany

2.1.7.b Phagemid vectors

pBluescript [®] II SK(+)	Stratagene, La Jolla, CA, USA
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2.1.7.c Filamentous phage

M13mp19 (Yanisch-Perron <i>et al.</i> 1985)	Boehringer Mannheim, Germany
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2.1.8 Tissue Culture

Basal Medium Eagle nutrient mix	CSL Ltd, Parkville, VIC, Australia
Dulbecco's phosphate buffered saline	Gibco BRL, Gaithersburg, MD, USA
F12 nutrient media	CSL Ltd, Parkville, VIC, Australia
Foetal bovine serum	CSL Ltd, Parkville, VIC, Australia
Trypsin	CSL Ltd, Parkville, VIC, Australia

2.1.9 Enzymatic Substrates

p-nitrocatechol sulphate	Sigma Chemical Co, St Louis, MO, USA
Cerebroside sulphate	Synthesised within the Department of Chemical Pathology as outlined by Poulos and Beckman (1985)
4-methylumbelliferyl-2-acetamido-2-deoxy-B-D-glucopyranoside (4MU)	Toronto Research Chemicals, Ontario, Canada

2.1.10 Kits and Miscellaneous Materials

3MM chromatography paper	Whatman, Maidstone, UK
Colony/plaque screen [™] hybridisation transfer membranes	NEN [®] DuPont, Boston, MA, USA
fmol [™] DNA Sequencing System	Promega Corp, Madison, WI, USA
DyeDeoxy [™] terminator sequencing kit	Applied Biosystems
Fuji RX medical X-ray film	Fuji Photo Film Co, Ltd
Gene Amp [™] PCR reaction tubes	Perkin Elmer, Norwalk, CT, USA
Herring sperm DNA	Boehringer Mannheim, Germany
Hybond [™] -N+ nylon membrane	Amersham, UK
Chelex 100 resin	Bio-Rad, 391 Park RD, Regents Park, NSW, Australia, 2143

Oligonucleotide purification cartridges	Perkin Elmer/Applied Biosystems, Foster City, CA, USA
Plasmid Midi Kit	QIAGEN Inc, Chatsworth, CA, USA
Positive land film, type 667	Polaroid (U.K.) Ltd., St Albans, Hertfordshire, England
Random Octomer oligonucleotides	Synthesised within the Department of Chemical Pathology, Women's and Children's Hospital, North Adelaide, SA
Tissue culture flasks	Corning Costar Corp. Cambridge, MA. USA
Sephadex G-50	Pharmacia Biotech, Uppsala, Sweden
Ultrafree [®] -MC, 0.45µm filter units	Millipore Corp, Bedford, MA, USA
Whatman filter paper, number 1	Whatman, Maidstone, UK

2.1.11 Miscellaneous Fine Chemicals

All chemicals used in this study were of analytical reagent grade and were purchased from a variety of manufacturers as follows:

Ammonium acetate	BDH Chemicals Australia Pty Ltd
Ammonium hydroxide	BDH Chemicals Australia Pty Ltd
Bacto-agar	Difco Laboratories, Detroit, MI, USA
Bacto-tryptone	Difco Laboratories, Detroit, MI, USA
Bacto-yeast extract	Difco Laboratories, Detroit, MI, USA
β-mercaptoethanol	Sigma Chemical Co, St Louis, MO
Boric acid	BDH Chemicals Australia Pty Ltd
Butan-1-ol	Ajax Chemicals Pty Ltd, Auburn, NSW, Australia
Calcium chloride	Ajax Chemicals Pty Ltd, Auburn, NSW, Australia
Chemicals for oligonucleotide synthesis	Applied Biosystems, Melbourne, Australia.
Chloroform	Ajax Chemicals Pty Ltd, Auburn, NSW, Australia
Deoxynucleotides	Boehringer Mannheim, Germany

Dideoxynucleotides	Boehringer Mannheim, Germany
Dimethylsulphoxide	Sigma Chemical Co, St Louis, MO, USA
D-glucose	BDH Chemicals Australia Pty Ltd
DTT	Gibco BRL, Gaithersburg, MD, USA
Ethanol	Ajax Chemicals Pty Ltd, Auburn, NSW, Australia
EDTA	BDH Chemicals Australia Pty Ltd
Glacial acetic acid	Ajax Chemicals Pty Ltd, Auburn, NSW, Australia
Guanidinium thiocyanate	Gibco BRL, Gaithersburg, MD, USA
Herring sperm DNA	Sigma Chemical Co, St Louis, MO, USA
Hydrochloric acid	Ajax Chemicals Pty Ltd, Auburn, NSW, Australia
Isoamyl alcohol (3-methyl-1-butanol)	J.T. Baker Chemical Co, Phillipsburg, NJ, USA
IPTG	Boehringer Mannheim, Germany
Magnesium chloride	Ajax Chemicals Pty Ltd, Auburn, NSW, Australia
Mineral oil	Sigma Chemical Co, St Louis, MO, USA
N-lauroylsarcosine (sarkosyl)	Sigma Chemical Co, St Louis, MO, USA
Phenol	Wako Chemical Co, Osaka, Japan
Potassium acetate	Ajax Chemicals Pty Ltd, Auburn, NSW, Australia
Potassium chloride	Ajax Chemicals Pty Ltd, Auburn, NSW, Australia
Propan-2-ol (isopropanol)	Ajax Chemicals Pty Ltd, Auburn, NSW, Australia
Sodium dodecyl sulphate	Sigma Chemical Co, St Louis, MO, USA
Sodium acetate	Ajax Chemicals Pty Ltd, Auburn, NSW, Australia
Sodium citrate	Ajax Chemicals Pty Ltd, Auburn, NSW, Australia
Sodium chloride	BDH Chemicals Australia Pty Ltd

Sodium hydroxide	Ajax Chemicals Pty Ltd, Auburn, NSW, Australia
Sodium pyrophosphate	Ajax Chemicals Pty Ltd, Auburn, NSW, Australia
Transfection reagent (DOTAP)	Boehringer Mannheim, Germany
Tris-HCl	Boehringer Mannheim, Germany

2.2 SOLUTIONS, BUFFERS AND MEDIA

2.2.1 Preparation of Glassware and Solutions

All solutions were prepared using Millipore water (0.04 uSiemens) and sterilised by autoclaving at 125°C and 120 kPa. Glassware was treated with Pyroneg detergent, rinsed in deionised water and dried overnight. Spatulas and centrifuge tubes were rinsed in ethanol and air dried. All restriction endonucleases and most other enzymes were provided with incubation buffers. Solutions and all other buffers routinely used in this study were prepared as follows:

Buffer saturated phenol	Wako phenol mixed with an equal volume of 50 mM Tris-HCl, pH 8.0
Ligation buffer	50 mM Tris-HCl pH 7.6, 10 mM MgCl ₂ , 5% (w/v) PEG 6000, 1 mM ATP, 1 mM DTT
Formamide loading buffer	96% (v/v) formamide, 20 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol
Lysis solution (DNA preparation)	0.32 mM sucrose, 10 mM Tris HCl pH 7.5, 5 mM, MgCl ₂ , 1% (v/v) Triton X-100
PCR buffer	67 mM Tris HCl pH 8.8, 16.6 mM (NH ₄) ₂ SO ₄ , 0.45% (v/v) Triton X-100, 0.2 mg/ml gelatin
Phosphate buffered saline	130 mM NaCl, 10 mM NaHPO ₄ , 10 mM NaH ₂ PO ₄ pH 7.2
1 x polynucleotide kinase buffer	50 mM Tris HCl pH 8.2, 10 mM MgCl ₂ , 0.1 mM EDTA, 5 mM DTT, 0.1 mM spermidine
Proteinase K buffer	10 mM Tris HCl pH 7.4, 5 mM MgCl ₂
Proteinase K working solution	40% (w/v) proteinase K, 300 mM NaCl, 30 mM sodium citrate

RNase working solution	50 mM Tris pH 7.5, 0.4 M NaCl, 10 mM EDTA, 0.5 U/μl RNase A, 0.025 U/μl RNase T1, 5% (v/v) glycerol
SSC	150 mM NaCl, 15 mM tri-sodium citrate.2H ₂ O, pH 7.0
T4 DNA ligase buffer	30 mM Tris HCl pH7.8, 10 mM MgCl ₂ , 10 mM, DTT, 0.5 mM ATP
TAE	0.4 M Tris HCl pH 7.8, 5 mM sodium acetate, 1 mM EDTA
TBE	0.89 M Tris HCl pH 8.3, 0.89 M boric acid, 25 mM EDTA
TE	10 mM Tris HCl, 0.1 mM EDTA pH 7.5.
Transfer solution	0.4 M NaOH, 0.6 M NaCl

2.2.2 Liquid Bacterial Media

Liquid media was prepared in Millipore water and sterilised by autoclaving immediately after preparation. The following media were used for the growth of all bacterial cultures within this study:

L-broth	1% (w/v) Bacto-tryptone, 1.0% (w/v) NaCl, 0.5% (w/v), Bacto yeast extract, pH 7.5 with NaOH
SOB media	2% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 0.05% (w/v) NaCl, 2.5 mM KCl, pH7.0
Minimal media	7.5 mM (NH ₄) ₂ SO ₄ , 16.5 mM KH ₂ PO ₄ , 30.1 mM K ₂ HPO ₄ , 0.85 mM sodium citrate

2.2.3 Solid Bacterial Media

L-agar	L-broth, containing 1% (w/v) Bacto agar
L Amp	L-broth, 1% (w/v) Bacto agar, 100 ug/ml ampicillin
SOB top agar	SOB media containing 0.8% (w/v) Bacto agar

Minimal media with glucose

Minimal media with 1% (w/v) Bacto agar,
0.02% (w/v) MgSO₄, 0.2% (w/v) glucose,
0.0005% (w/v) thiamine

2.3 GENERAL METHODS

2.3.1 Preparation of Genomic DNA

Whole blood samples (20 ml) were mixed with 30 ml of lysis buffer and left to stand for 30 min before centrifugation at 2000 g for 5 min at 10°C. All but 5 ml of the supernatant was removed and a further 30 ml of lysis buffer was added to the pellet, the cell suspension was mixed and the centrifugation repeated. The supernatant was removed leaving the pellet as dry as possible. The pellet was resuspended in 3 ml of proteinase K buffer containing 2.8% (v/v) SDS and 200 µl of 10 mg/ml proteinase K. This solution was incubated overnight at room temperature on a rotating platform, then extracted twice with buffer saturated phenol and once with chloroform/isoamyl alcohol (1:1) before ethanol precipitation of the DNA.

2.3.2 Ethanol Precipitation of DNA

Genomic and plasmid DNA samples were precipitated in ethanol in the presence of 0.3 M sodium acetate pH 5.5 or 3.75 M Ammonium acetate and two volumes of 100% ethanol for 1 hr at -20°C unless otherwise stated. Following centrifugation, DNA pellets were rinsed in 70% ethanol before being dried briefly under vacuum and resuspended in a minimal volume of dH₂O or TE buffer.

2.3.3 Restriction Endonuclease Digestion of DNA

All restriction endonuclease digestions were carried out using the conditions specific for each individual enzyme as recommended by the manufacturer (Boehringer Mannheim).

2.3.4 Electrophoresis

2.3.4.a Agarose gel electrophoresis

Electrophoresis of DNA for analytical purposes was carried out in either Pharmacia GNA100 or GNA200 submarine gel tanks depending on the number of samples to be analysed. Gels were made of 1% (w/v) agarose in 1 x TBE buffer unless otherwise stated. Samples were electrophoresed so as to sufficiently separate DNA fragments of interest. DNA was visualised under UV (306 nm) light after staining of the gel in 10 ug/ml ethidium bromide. A photograph was taken using positive land film 667.

2.3.4.b Polyacrylamide gel electrophoresis

Denaturing polyacrylamide gels were prepared using commercially available solutions and polymerised according to the manufacturers instructions (National Diagnostics). Typically, 6% w/v (19:1) polyacrylamide, 7 M urea, 1 x TBE gels were used and electrophoresed at a constant voltage of 1850 V in IBI gel tanks to sufficiently separate bands of interest. After electrophoresis, gels were fixed in 20% (v/v) EtOH, 10% (v/v) glacial acetic acid, transferred to Whatman[®] 3MM paper and vacuum dried before exposure to X-ray film.

Non-denaturing polyacrylamide gels were prepared from solid acrylamide and bis-acrylamide in the ratios required. The polyacrylamide solution was degassed under vacuum for 60 min and deionised for 60 min using Chelex 100 resin. Following filtration, through Whatman number. 1 filter paper, the polyacrylamide solution was stored at 4°C until required. Non-denaturing gels contained no urea and were electrophoresed at a low voltage (typically 650-750 V) which produced no excess heat. The gels were not fixed, but were vacuum dried and exposed to autoradiographic film as previously stated.

2.3.5 Synthesis of DNA Oligonucleotides

All oligonucleotides used in this study were synthesised on an Applied Biosystems 391 DNA synthesiser or a Beckman Oligo 1000 synthesiser within the department of Chemical Pathology at the Women's and Children's Hospital. All oligonucleotides were designed specifically for this study except where referenced. All oligonucleotide sequences are listed in Appendices 3.1 to 3.3 and are discussed within relevant parts of the text of this thesis.

2.3.6 Purification of Oligonucleotides

2.3.6.a Method 1: n-butanol extraction

Oligonucleotides less than 40 bp in length were cleaved from their synthesis columns by the addition of 1.0 ml of 10 M NH_4OH and incubation for 60 min at room temperature. The cleaved oligonucleotides were then incubated at 55°C overnight to remove protection groups then purified using the protocol of Sawadogo and Van Dyke (1991). A 100 μl sample of deprotected oligonucleotide was mixed with 1 ml of *n*-butanol and the mixture centrifuged at 13000 g for 5 min at room temperature. The resultant pellet was resuspended in 100 μl water and the butanol extraction repeated. The final pellet was resuspended in water and the volume adjusted to give a working concentration of 100 $\mu\text{g}/\mu\text{l}$.

2.3.6.b Method 2: OPC purification

Oligonucleotides greater than 40 bp in length were purified and desalted with oligonucleotide purification cartridges using the methods and solutions supplied by the manufacturer (Applied Biosystems).

2.3.7 The Polymerase Chain Reaction (PCR)

Amplification of genomic DNA, plasmid DNA and cDNA were performed as outlined by Saiki *et al.* (1988). Each reaction contained 2.5 to 3.5 mM MgCl₂, 1 x Biotech International PCR buffer, 400 μ M dNTPs, 100 ng of each primer, 1.2 U of *Taq* DNA polymerase and approximately 100 μ g of target DNA. Amplifications of cDNA and plasmid DNA also contained 4% (v/v) DMSO. PCR cycling reactions were performed with varying times and temperatures for denaturation, annealing and extension, depending on the product being amplified and the primers used as outlined in the appropriate sections of the text of this thesis. Appropriate positive and negative controls were performed in parallel with all experimental PCR reactions.

2.3.8 5'-End Labelling of Oligonucleotides

Synthetic oligonucleotides were 5' end labelled using T4 DNA polynucleotide kinase and [γ ³²P] ATP as described by Chaconas and van de Saude (1980).

2.3.9 Transfer of DNA onto Nylon Membranes

2.3.9.a Southern blotting

Restriction endonuclease digested DNA or PCR amplified DNA was size fractionated on agarose gels and transferred onto HybondTM-N⁺ nylon membranes using the alkaline transfer method as outlined by Reed and Mann (1985). The agarose gel was soaked in transfer solution for 15 min with gentle shaking prior to transfer. Nylon membrane was cut to the size of the gel and also soaked in transfer solution. The gel was placed on a wick of Whatman[®] 3MM paper and the membrane placed on top of the gel followed by two sheets of 3MM paper and blotting tissue. Transfer was carried out for at least 4 hrs. Following transfer, the blot was disassembled and the membrane neutralised in 1.0 M Tris HCl pH 7.5, 0.6 M NaCl for 10 min before being air dried.

2.3.9.b *Plaque lifting*

Plaques which had grown overnight on L-agar plates in a SOB overlay were placed at 4°C for at least 60 min prior to lifting. Colony/Plaque Screen™ membranes were gently placed on the plates and left at room temperature for 2 min for the first lift and 4 min for subsequent duplicates, following which, the membranes were carefully removed from the plates and placed 'plaque side up' on a pool (5 ml) of 1.5 M NaCl, 0.5 M NaOH for 7 min. The membranes were neutralised by placing the filters plaque side up onto 1.0 M Tris HCl pH 7.5, 1.5 M NaCl, 1.0 mM EDTA for 3 min, which was repeated with fresh neutralising solution. Membranes were briefly rinsed in 2 x SSC, drained and allowed to air dry. DNA was fixed to the filters by placing the dry filter plaque side up on a pool (10 ml) of 0.4 M NaOH for 15 min. The membranes were briefly rinsed in 5 x SSC and allowed to air dry completely before storage at 4°C.

2.3.10 Prehybridisation, Hybridisation and Washing of Filters

Prehybridisation of southern blots or plaque lifts was carried out at 45°C, unless otherwise stated, in a solution of 6 x SSC, 0.05% (w/v) sodium pyrophosphate, 0.1% (w/v) SDS and 100 µg/ml denatured herring sperm DNA for at least 60 min in a shaking water bath. Approximately 50 ng of 5' end-labelled oligonucleotide probe was added to the prehybridisation solution and filters were hybridised for at least 3 hrs at 45°C unless otherwise stated.

Hybridised filters were initially rinsed twice in 6 x SSC for 10 min, followed by a critical wash in 2 x SSC, 0.1% SDS. The temperature of the critical wash solution was dependent on the sequence of the oligonucleotide probe. The wash temperature was slowly increased until all non-specifically bound probe was removed. Washed filters were exposed to Fuji RX X-ray film at room temperature or at -80°C in the presence of tungsten intensifying

screens if low levels of radioactivity were to be detected. The sequence and specific washing temperatures of all oligonucleotide probes used in this study appear in Appendices 3.1 and 3.4 and are discussed in relevant parts of the text within this thesis.

2.3.11 Dideoxy Sequencing

All plasmid and PCR products in this study were sequenced using a *fmol*TM DNA Sequencing System using the solutions and methods recommended by the supplier (Promega Corp) unless otherwise stated. Sequencing products were electrophoresed under denaturing conditions as outlined in Section 2.3.4 *b*. Automated sequencing was performed using an Applied Biosystems, DyeDeoxyTM terminator sequencing kit with the materials and methods supplied by the manufacturer. Samples were analysed on an Applied Biosystems 373 sequencer in the Department of Cytogenetics, Women's and Children's Hospital, North Adelaide, South Australia.

2.3.12 Preparation of Plasmid DNA

2.3.12.a Small scale plasmid preparations

Plasmid or M13 replicative form (RF) DNA minipreps were prepared using a modified alkaline lysis protocol (Sambrook *et al.*, 1989). Briefly, single cell colonies were grown overnight in 2 ml LB cultures containing the appropriate antibiotic. Cells were pelleted at 13000 g for 2 min at 4°C and resuspended in 100 µl of ice cold 25 mM Tris HCl pH 8.0, 10 mM EDTA and 50 mM glucose. Freshly prepared 0.2 M NaOH, 1% (w/v) SDS (200 µl) was added to each tube, mixed by inversion and allowed to incubate at room temperature for 5 min. Ice-cold 3 M potassium acetate (150 µl) was added to each preparation, the tube was then vortexed in an inverted position and incubated on ice for 15 min. Cellular debris and chromosomal DNA was pelleted at 13,000 g for 10 min at 4°C. The resultant supernatant was

extracted with an equal volume of buffer saturated phenol:chloroform (1:1) before the plasmid DNA was precipitated by the addition of EtOH and resuspended in 50 ul H₂O.

2.3.12.b Midi scale plasmid preparations

Midipreps of plasmid DNA or M13-RF DNA were performed using QIAGEN Plasmid Mini Kits using the solutions and protocols supplied by the manufacturer.

2.3.13 Cloning into Plasmid Vectors

2.3.13.a Preparation of plasmid vectors

Vector and insert DNAs for use in ligation reactions were restricted with appropriate restriction endonuclease(s) under conditions recommended by the manufacturer. Vector DNA which was cut with a single enzyme, was treated with calf intestinal phosphatase according to the manufacturers instructions (Boehringer Mannheim) in order to prevent religation of the vector. Vector and insert bands were size fractionated by electrophoresis in 1% (w/v) agarose gels and purified from the agarose using Ultrafree[®]-MC 0.45 um filter units by a freeze-squeeze method outlined by Thuring *et al.* (1990). The DNA was resuspended in H₂O to a working concentration of 5-20 ng/ul.

2.3.13.b Preparation of PCR generated inserts

PCR products to be cloned into the pGEM[®]-T PCR cloning vector were used directly from the PCR mix without further purification.

2.3.13.c Ligation reactions

Vector and insert DNAs to be ligated were mixed in an 8 ul reaction volume containing a total of approximately 60 ng of DNA, with the insert DNA being in a 3 molar excess. The vector/insert mix was heated to 65°C for 3 min before being chilled on ice. Following addition of 1 ul of 10 x T4 DNA ligase buffer and 1 ul of T4 DNA ligase (1-3

U/ul), the ligation mixture was incubated for 3 hrs at 16°C before an overnight incubation at 4°C.

2.3.13.d Preparation and transformation of *E. coli*

E. coli-cells were made competent using the following method. L-broth media (50 ml) was inoculated with 2 ml of an overnight culture of *E. coli*-cells grown from a single colony (Sambrook *et al.*, 1989). The inoculated culture was incubated at 37°C on a shaking platform until the cells had grown to an A₆₀₀ of 0.4 to 0.6. The cells were harvested at 2000 g for 5 min at 4°C and resuspended in 10 ml of ice cold 0.1 M MgCl₂ before incubation on ice for 30 min. The cells were harvested as before and the resultant pellet resuspended in 10 ml of ice cold 0.1 M CaCl₂ and allowed to incubate on ice for at least 2 hr. The cells were again harvested by centrifugation as before and resuspended in 1.6 ml of ice cold 0.1 M CaCl₂, 20% (v/v) glycerol and snap frozen in 100 ul aliquots by immersion in liquid nitrogen. Competent cells were stored at -80°C until required.

For transformation, competent cells were allowed to thaw on ice before the addition of the 10 ul ligation mixture. Plasmid DNA and competent cells were mixed gently and incubated on ice for 2 hours prior to heat shocking at 37°C for 5 min and 45°C for 90 sec for *E. coli* MC1061 and *E. coli* DH5αF' strains respectively. After heat shock, the cells were returned to ice for 2 min before the addition of 300 ul of L-broth and incubation at 37°C for 60 min with shaking. The transformation mix was plated onto L-amp plates and incubated at 37°C overnight.

RF M13 plasmids were transformed into DH5αF' cells as above. After heat shock, the cells and 50 ul of an overnight culture of DH5αF' plating cells were added to 8 ml of melted SOB agar stored at 50°C. The mixture was vortexed briefly then poured over a 10 cm

L-agar plate, allowed to set and incubated at 37°C overnight as described by Sambrook *et al.* (1989).

2.3.14 Enzymatic Assays

2.3.14.a Assay of arylsulphatase A activity

Arylsulphatase A activity was assayed in CHO cell expression lines, peripheral leukocytes and cultured skin fibroblasts as previously described by Baum *et al.* (1959). ASA activity toward the natural substrate, cerebroside sulphate, was assayed as described by Poulos and Beckman (1985).

2.3.14.b The assay of lysosomal fractions

For the assay of ASA activity, 5 ul from each fraction obtained from Percoll[®]/sucrose density gradients was diluted in 95 ul of 0.45% (w/v) NaCl. This solution was assayed as previously described by Baum *et al.* (1959). β -hexosaminidase activity was measured in a 5 ul aliquot of each density fraction diluted in 45 ul of 0.9% (w/v) NaCl as described by Leabeck and Walker (1961).

2.3.15 Tissue Culture

CHO and skin fibroblast cell lines were maintained on HAMS F12 and Basal Medium Eagle nutrient media containing 10% (v/v) foetal bovine serum and 1% (v/v) penicillin/streptomycin respectively. Confluent flasks of tissue culture cells were subcultured by removing the nutrient media from the tissue culture flasks and rinsing the cells with 5 ml PBS. The PBS was removed and 3 ml of 10% (v/v) (CHO cell lines) or neat (skin fibroblast lines) trypsin was added and allowed to incubate at room temperature for 5 min. The cultured cells were subsequently “knocked” to release them from the bottom of the tissue culture flasks

and into suspension, 1/4 of the trypsin/culture cell volume was removed and added to fresh culturing media. Cells were maintained at 37⁰C in an atmosphere of 5% (v/v) CO₂.

Chapter 3

Genotype/Phenotype Correlation in Metachromatic Leukodystrophy

3.1 INTRODUCTION

3.1.1 Opening Remarks

McKusick (1973) was first to hypothesise that there was a link between allelic mutations, varying deficiencies of an enzyme product and apparent diversity in the presentation of a genetic disease. It is now well accepted however, that the clinical variation observed in the presentation of many inborn errors of metabolism is due to the existence of multiple disease causing mutations, each of which result in different levels of enzyme deficiency. McKusick *et al.* (1972) used the term “allelic series” to collectively describe the array of different alterations that could be detected within a particular gene and used this term when hypothesising that the clinically distinct Hurler and Scheie syndromes were, in fact, caused by different allelic mutations within an unidentified (at that time) gene, since both disorders demonstrated a biochemical deficiency of the same enzyme product. Different mutations at this unidentified locus were thought to lead to varying deficiencies of the same enzyme, which subsequently resulted in the presentation of distinct clinical phenotypes.

3.1.2 Specific Aim

This chapter presents the results of the search for MLD mutations within the ASA gene. Twenty nine MLD patients and twenty one individuals with a pseudodeficient level of ASA activity were screened for previously identified mutations. A subset of MLD patients in which no ASA mutations were identified, were subsequently used in a search for novel disease causing mutations. The aim of this analysis, was to correlate patient genotypes with clinical presentation, with a view to predicting the severity of disease associated with the presence of different ASA mutations, particularly when patients are compound heterozygotes for different MLD alleles.

3.2 SPECIFIC MATERIALS AND METHODS

3.2.1 Specific Materials

3.2.1.a Enzymes

Mo-MLV reverse transcriptase	Gibco BRL, Gaithersburg, MD, USA
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3.2.1.b Radiochemicals

[α - ³² P]dCTP (6000Ci/mmol)	NEN [®] DuPont, Boston, MA, USA
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3.2.1.c Solutions and Buffers

First strand reverse transcriptase buffer	50 mM Tris HCl pH 8.3, 75 mM KCl, 3 mM MgCl ₂
---	--

Solution D	4 M Guanidinium thiocyanate, 25 mM sodium citrate, 0.5% (w/v) sarkosyl, 0.1 M β -mercaptoethanol, pH 7.0
------------	--

3.2.2 Specific Methods

3.2.2.a Isolation of RNA

Total RNA was extracted from cultured skin fibroblasts using a method adapted from Chomczynski and Sacchi (1987). Briefly, pelleted skin fibroblasts from a confluent T70 tissue culture flask (approximately 15 mg of cells) were resuspended in 350 μ l of solution D, to which was sequentially added, 35 μ l 2 M sodium citrate, 350 μ l of water-saturated phenol and 70 μ l of chloroform:isoamyl alcohol (49:1). The suspension was shaken vigorously for 10 sec and incubated on ice for 60 min before centrifugation at 13000 g for 20 min at 4°C. The aqueous phase was retained and mixed with 350 μ l of isopropanol before incubation at -20°C overnight. The RNA was recovered by centrifugation at 13000 g for 20 min at 4°C. The resulting pellet was resuspended in 100 μ l of solution D and the RNA reprecipitated by

mixing with 200 μ l of isopropanol and incubating at -20°C for 60 min. RNA was pelleted as before and rinsed in 70% EtOH before being air dried and resuspended in 50 μ l of distilled water. All RNA samples were stored at -80°C .

3.2.2.b *cDNA synthesis*

A sample of total RNA (3 μ g) was combined with 1 μ l RNasin[®], 5 μ l of DDT, 5 μ l of 5 x BRL reverse transcriptase buffer and 5.2 μ l of 500 ng/ μ l random octomers in a final volume of 46 μ l. This solution was incubated at 65°C for 3 min. After cooling on ice, 3 μ l (200U) of reverse transcriptase and 1 μ l of 25 mM dNTPs were added and the mixture incubated for 60 min at 37°C followed by a further 60 min incubation at 37°C after the addition of 5 μ l of 3 M sodium hydroxide. The mix was neutralised with 1.25 μ l of concentrated HCl before the cDNA was precipitated by the addition of EtOH and resuspended in 50 μ l H₂O.

3.2.2.c *SSCP analysis of genomic and cDNA copies of ASA*

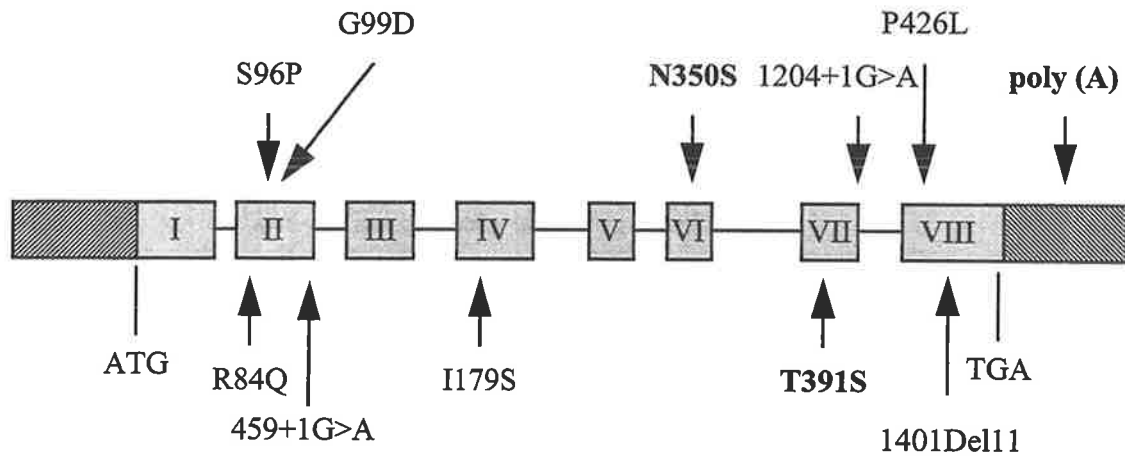
SSCP analysis was performed as outlined by Orita *et al.* (1989). PCR reactions were performed under conditions specific for primer pairs as presented in Appendices 3.2 and 3.3, in the presence of 1 μ l of 10 mCi/ml [$\alpha^{32}\text{P}$]dCTP. The PCR product (3 μ l) was diluted in 50 μ l of 0.1% (w/v) SDS and 10 mM EDTA and 5 μ l of the diluent was mixed with 5 μ l of formamide loading buffer. After denaturation at 96°C for 3 min, 4 μ l samples of this mixture were loaded onto a non-denaturing 6% (19:1), 10% (v/v) glycerol, 0.5 x TBE and a 10% (49:1), 10% (v/v) glycerol, 1 x TBE polyacrylamide gel. The two gels were run at 30 W and 20 W constant power for 6 and 12 hrs respectively on Pharmacia sequencing apparatus. The gels were vacuum dried and exposed to Fuji RX X-ray film for 24 to 48 hrs without intensifying screens.

3.3 RESULTS AND DISCUSSION

3.3.1 Screening For Previously Identified MLD Alleles

Eight MLD mutations and three non-pathogenic sequence variants, N350S, T391S and the ASA-PD polyadenylation defect, had been identified within the ASA gene prior to the commencement of this project (Figure 3.1). Allele specific oligonucleotides (ASOs) were used to detect carriers of these alterations within a population of patients (n=29) who were clinically and biochemically diagnosed as having MLD. The results of this analysis are summarised in Table 3.1. Twenty one individuals who were identified as having pseudodeficient levels of ASA activity were also screened for the previously identified MLD alleles, as summarised in Table 3.2. The clinical presentation of these individuals to the Women's and Children's Hospital warranted an investigation for MLD, but their symptoms were subsequently found to be unrelated to their low 'pseudodeficient levels' of ASA activity. The ASOs used in the study appear in Appendix 1 as do the critical washing conditions under which they were used. ASOs were hybridised to PCR products amplified from the genomic DNA of each MLD patient and ASA pseudodeficient individual. The oligonucleotides used to amplify the ASA exons and intron boundaries, together with PCR conditions, appear in Appendix 2.

The measured frequency of the common late-infantile MLD mutation (459+1G>A) in the Australian MLD population (27.5%) was similar to that reported by Polten *et al.* (1991) who identified the mutation in approximately 25% of MLD alleles. The common adult mutation P426L was, however, found to be relatively less frequent in the Australian MLD population. The P426L mutation was found on only 7% of the Australian MLD alleles while being reported to occur with an allele frequency of 25% by Polten *et al.* (1991). The reason for this difference and its importance is unclear.



Figures 3.1 Screening for previously identified ASA mutations

The eight mutations in the human ASA gene identified prior to the commencement of this project are presented as are three non-pathogenic ASA sequence variants (bold). Shaded areas represent the eight ASA exons which are numbered and hatched areas the 5' and 3' untranslated regions of the gene. The initiation and termination codons are marked.

Patient	ASA-PD	459+1G>A	P426L	I179S	1204+1G>A	1401Del11	G99D	S96P	R84Q	N350S	T391S
PS	--	+-	--	--	--	--	--	--	--	--	--
SN	+-	--	--	--	--	--	--	+-	--	+-	--
MM	++	--	--	--	--	--	--	--	--	++	--
AW	--	+-	+-	--	--	--	--	--	--	--	--
NM	--	++	--	--	--	--	--	--	--	--	--
LP	--	--	--	--	--	--	--	--	--	--	--
BA	--	+-	--	--	--	--	--	--	--	--	--
SA	--	--	--	--	--	--	--	--	--	--	--
TB	--	++	--	--	--	--	--	--	--	--	--
PE	--	--	--	--	--	--	--	--	--	--	--
MG	--	--	--	--	--	--	--	--	--	--	--
CG	--	--	--	--	--	--	--	--	--	--	--
SG	--	--	--	--	--	--	--	--	--	--	--
SB	--	--	--	--	--	--	--	--	--	--	--
RK	--	+-	--	+-	--	--	--	--	--	--	--
MG	--	+-	+-	--	--	--	--	--	--	--	--
PG	++	--	--	--	--	--	--	--	--	++	--
CH	--	+-	--	--	--	--	--	--	--	--	--
LH	--	--	+-	--	--	--	--	--	--	--	--
AK	--	--	--	--	--	--	--	--	--	--	--
KN	--	--	--	--	--	--	--	--	--	--	--
JR	+-	--	--	--	--	--	--	--	+-	+-	--
DW	--	+-	--	--	--	--	--	--	--	--	--
WT	++	--	--	--	--	--	--	--	--	++	--
GS	+-	--	--	--	--	--	--	--	--	+-	--
ST	--	+-	--	--	--	--	--	--	--	--	--
CS	--	+-	--	+-	--	--	--	--	--	+-	--
KT	--	+-	+-	--	--	--	--	--	--	--	--
RH	--	++	--	--	--	--	--	--	--	--	--

Table 3.1 ASO screen for known ASA mutations in the MLD patient population

Twenty nine MLD patients were screened for nine MLD mutations as well as the non-pathogenic ASA-PD polyadenylation defect, N350S and T391S amino acid substitutions (“+” indicates the presence of the mutant allele and “-” indicates that the normal ASA allele is present). The ASA-PD, 459+1G>A and P426L ASO screens were performed by Mr Paul Nelson, Department of Chemical Pathology, Women’s and Children’s Hospital, Adelaide, South Australia, as part of the department’s diagnostic service.

<u>Patient</u>	<u>ASA-PD</u>	<u>N350S</u>	<u>T391S</u>	<u>W193C</u>
LJ	++	++	--	--
KK	++	++	--	--
AS	+-	+-	+-	+-
HM	++	++	--	--
PG	+-	+-	+-	+-
JR	++	++	--	--
SP	+-	+-	+-	+-
AK	++	++	--	--
RW	+-	+-	+-	--
MC	++	++	--	--
LS	+-	+-	--	--
AW	++	++	--	--
JG	+-	+-	+-	--
KP	++	++	--	--
KO	++	++	--	--
NZ	+-	+-	--	--
AJ	++	++	--	--
MP	++	++	--	--
DG	+-	++	+-	--
NW	++	--	++	--
MS	++	++	--	--

Table 3.2 ASO screen for known MLD mutations in the ASA-PD population

The ASA pseudodeficiency population was screened for all mutations analysed in Table 3.1. Only alleles for which the population was positive appear on the above Table, (“+” indicates the presence of the mutant allele and “-” the normal ASA allele). The pseudodeficiency population was screened for W193C by Ms. Kelly Perkins, Department of Chemical Pathology, Women’s and Children’s Hospital, Adelaide, South Australia.

It is not surprising that rare alleles, previously identified in only single MLD patients, were not identified in the Australian MLD population. On the contrary, it is almost surprising to find examples of these alleles (one R84Q and one S96F heterozygote) since such rare mutations are often restricted to single pedigrees (Gieselmann *et al.*, 1994). In this study, the R84Q and S96F mutations were both identified in late-infantile MLD patients. Consequently, these mutations would be expected to be classified as severe, yet they were previously reported to occur in juvenile (Kappler *et al.*, 1992) and late-infantile (Gieselmann *et al.*, 1991) MLD patients respectively.

The identification of the R84Q mutation in two clinically distinct patients may seem discrepant, yet it must be remembered that clinical presentation is the product of the residual activities of two MLD mutations. The identification of a juvenile R84Q heterozygote by Kappler *et al.* (1992) could therefore be accounted for by the combination of this mutation with a mild adult allele. The patient in question (JR) was in fact, found to be a compound heterozygote for the adult mutation, P426L and R84Q.

The detection of two I179S heterozygotes in the MLD patient population is interesting since these individuals represent the only examples of adult MLD which have been clinically diagnosed in Australia (patients CS and RK). Both of the individuals were found to be compound heterozygotes for the common late-infantile allele, 459+1G>A and I179S. The two patients both presented in their teens and were still alive in their late twenties.

Compound heterozygotes for the late-infantile, 459+1G>A and adult, P426L mutations present with the intermediate, juvenile form of MLD (Polten *et al.*, 1991). It is therefore reasonable to assume, that the I179S mutation expresses a higher level of residual ASA activity than that of P426L, since I179S/459+1G>A compound heterozygotes present with a milder adult form of the disease.

It remains unclear whether the I179S allele is pathogenic in the homozygous form, since no such individuals have been reported either in this study or in the literature. Fluharty *et al.* (1991) transiently expressed the I179S mutation and showed that it produced a residual ASA enzyme activity of about 5% of normal. This is a level of activity comparable to that estimated to occur in compound heterozygotes for the ASA-PD allele and the 459+1G>A late-infantile mutation. These individuals are clinically normal (Penzein *et al.*, 1993). Regardless of the relative pathogenicity of the I179S allele, it is clear that in the Australian MLD population, its presence is responsible for the presentation of MLD patients with the mildest form of the disease.

The screening of the MLD and pseudodeficient populations for the ASA-PD allele also yielded results of interest (Table 3.2). As expected most pseudodeficient individuals (62%) were found to be homozygous for the ASA-PD allele, but the remaining individuals were only carriers. The pseudodeficient patients have invariably presented with clinical symptoms suggesting a diagnosis of MLD, yet, upon investigation, it was found that they had ASA activities significantly above those seen in MLD patients. It was therefore concluded that the symptoms seen in these patients were unrelated to lowered ASA activity levels. All of these individuals, including those who were only heterozygous for the ASA-PD allele, had ASA activities in the pseudodeficiency range suggesting that other ASA pseudodeficiency alleles may exist apart from the common allele which contains the poly (A) and N350S sequence variants (see section 1.3.4).

Interestingly, a significant proportion of patients with only a single copy of the ASA-PD allele containing the polyadenylation defect and N350S had inherited additional alterations within the ASA gene. These include the W193C and T391S sequence variants and N350S in isolation (Table 3.2), all which have been previously reported to have no effect on the activity of the ASA enzyme (Polten *et al.*, 1991, Gieselmann *et al.*, 1989). The additional

mutations identified in these patients have not been assigned to alleles and their inheritance by these individual may have occurred by chance, but the observation raises the question of whether sequence variants like N350S, T391S and W193C have the ability to subtly reduce the activity of the ASA enzyme even though they had previously been thought to be benign. In support of this idea, results presented in Chapters 4 and 5 revealed that N350S and T391S do significantly affect ASA activity.

Within the MLD patient population, three homozygous and three heterozygous ASA-PD patients were identified. Given the carrier frequency of the ASA-PD allele in the Australian population of 1 in 5 (Nelson *et al.*, 1991) it is not surprising to identify the allele within the MLD patient group. The identification of nine ASA-PD alleles in 58 (Table 3.1) does, however, give a carrier frequency of ASA-PD allele in the MLD patient population of 1 in 3. Although not statistically significant ($\chi^2_1=1.76$) it unclear whether the difference in the carrier frequency of ASA-PD in the normal and MLD populations is significant in the causation of disease.

3.3.2 The Identification of Novel ASA Sequence Variants

Of the 29 MLD patients screened for known mutations, no disease causing mutations were identified in 13 individuals (this group included the three homozygotes and two heterozygotes for the ASA-PD allele; Table 3.1). These patients were used in a search for novel pathogenic alleles, since they represented the group in which the identification of such alleles was most likely.

Single strand conformation polymorphism (SSCP) analysis was used to identify regions of the ASA genomic and cDNA sequences which contained sequence alterations, as outlined in Figure 3.2. Oligonucleotide primers used to amplify genomic fragments of the ASA gene appear in Appendix 2. Those used to amplify cDNA copies of the gene appear in

Figure 3.2 SSCP analysis of the ASA gene

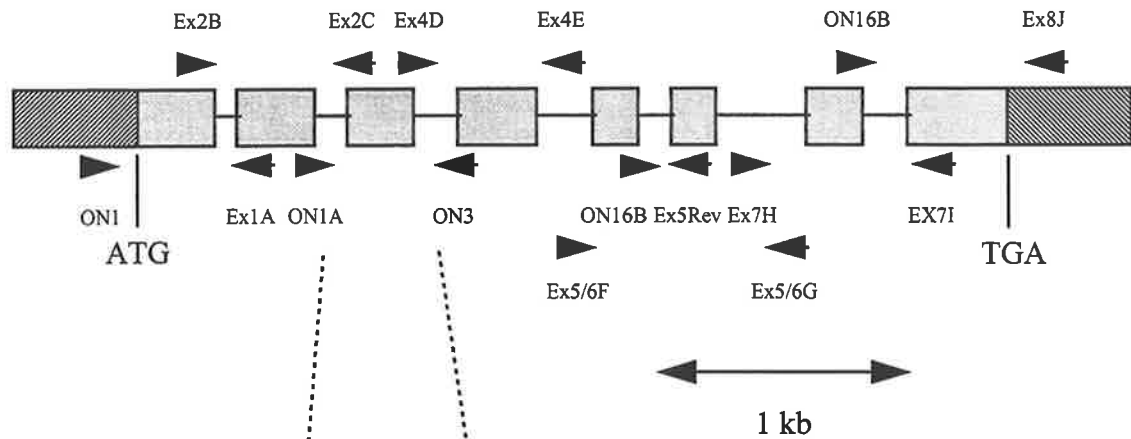
a) Schematic diagram of the ASA gene, showing the approximate binding position of the genomic ASA PCR primers together with the initiation and termination codons of the gene. Blocked areas represent the eight ASA exons and hatched areas the 5' and 3' untranslated regions of the gene

b) Amplification of exon 3 of the ASA gene using primers ON1A and ON3. Arrows represent the position of a hypothetical heterozygous base change to demonstrate the process of SSCP detection of sequence alterations.

c) Labelled PCR products were heat denatured and the single stranded products were allowed renature to form a secondary structure. The structure or conformation produced is a product of the base sequence of the single stranded fragment.

d) An example of electrophoresis of conformation products under non-denaturing conditions. The speed that a product runs through the polyacrylamide gel is governed by its secondary structure or conformation. Fragments differing by a single base may show altered conformation and hence altered mobility.

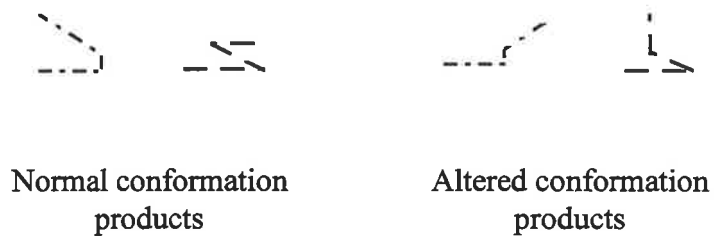
a)



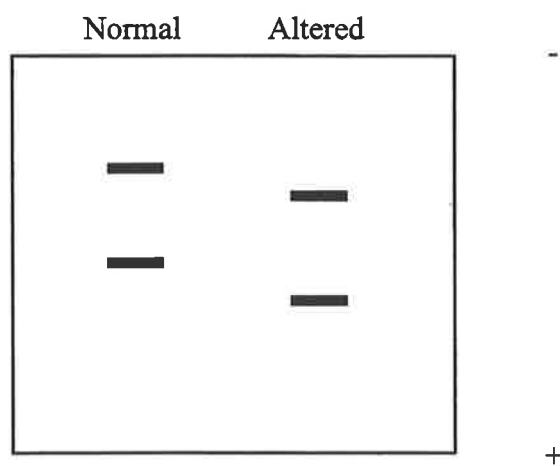
b)



c) Denaturation and formation of conformation products



d) PAG electrophoresis and autoradiography



Appendix 3 as do the conditions under which they were used. SSCP fragments with altered mobility (in comparison to normal controls) were sequenced (as described in section 2.3.11) to identify the cause of the conformational change. The presence of novel sequence variants identified in the patient population was confirmed by ASO analysis (Appendix 4). The patient (n=29), ASA-pseudodeficient (n=21) and normal control populations (typically, n=50) were then screened for the novel alleles with ASOs encoding the normal and mutant sequence of each sequence variant.

3.3.2.a MLD in the Australian Lebanese community

SSCP analysis of exon 4 genomic PCR products revealed that three MLD patients had a conformational alteration in the mobility of both bands and that a different individual was heterozygous for a similar change (Figure 3.3a). Sequence analysis of amplified exon 4 DNA from a normal control and samples that showed altered SSCP mobility revealed that the three MLD patients identified by SSCP had a single (C>T) transition which altered an ACG codon to an ATG codon. This resulted in the substitution of a threonine residue at amino acid number 274 by a methionine (T274M) (Figure 3.3b). No other SSCP changes were detected in the genomic or cDNA copies of the ASA gene of these patients.

Allele specific oligonucleotides were designed to detect the normal and mutant sequences of T274M (Figure 3.3c). The remaining MLD patients, ASA pseudodeficiency and normal control populations (n=66) were screened for the novel allele. ASO analysis revealed that a total of six MLD patients were homozygous for the T274M sequence variant, of whom three were sibs. No carriers were found in the normal population. All available parents of these six children, (n=6) were found to be heterozygous for T274M.

When the sequences of ten related sulphatase enzymes from a diverse range of species were compared, ASA-T274 was one of only 8 amino acids (out of 507) that were strictly conserved among the related enzymes (Figure 3.4). In addition, this residue was in the region of two further invariant residues. It is expected that a mutation in such a conserved residue would result in the production of ASA which either lacked catalytic activity or was unstable. This is consistent with the very low ASA activity measured in blood leukocyte and skinfibroblasts of all six of these patients (data not shown) and their uniform presentation with symptoms at the severe end of the clinical spectrum.

The apparently high allele frequency of the novel T274M mutation in the MLD population (20%, or 12 out of 58 alleles) may not reflect its true frequency in our general MLD patient group because of the high degree of consanguinity and the common ethnic origin of these patients. All of these patients are from four families of Lebanese extraction and all involve consanguineous marriages. In addition, at least two of the MLD families are closely related as shown by the pedigree in Figure 3.5. The T274M mutation is undoubtedly a very common allele affecting most (6 out of 7 patients) Australian MLD patients of Lebanese or Arabic descent. The high frequency of the T274M mutation among the Australian MLD population also goes some way to explaining the relatively low population frequency of the adult P426L mutation (see section 3.3.1), since the exclusion of individuals with the novel T274M mutation would result in an allele frequency of about 9% for the P426L MLD allele.

The identification of the T274M mutation in the Lebanese MLD patients has shed new light on a previous diagnostic dilemma. Three of the T274M homozygotes were sibs and all were similarly affected with severe late-infantile MLD (data not shown). A subsequent prenatal sample was incorrectly diagnosed as being affected. Accurate diagnosis in this case (prior to the isolation of the ASA gene) was difficult because as well as carrying the T274M MLD mutation, members of this family also carried the ASA-PD allele (Figure 3.6). As

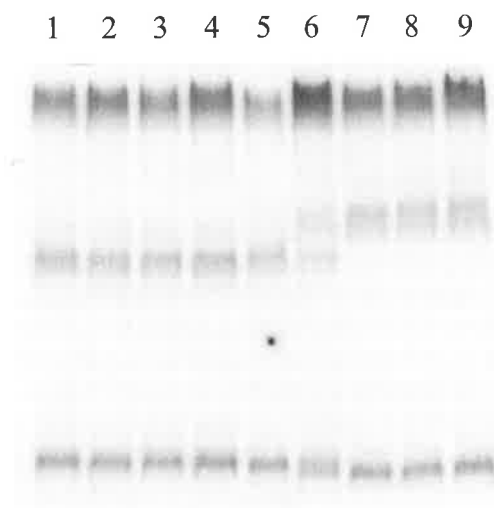
Figure 3.3 SSCP and sequence analysis of exon 4

a) SSCP analysis of exon 4 ASA genomic PCR products. Tracks 1-5 are controls, tracks 7-9 are from MLD patients who are homozygous for a similar SSCP alteration. Track 6 is from an individual who was heterozygous for normal and altered conformation bands.

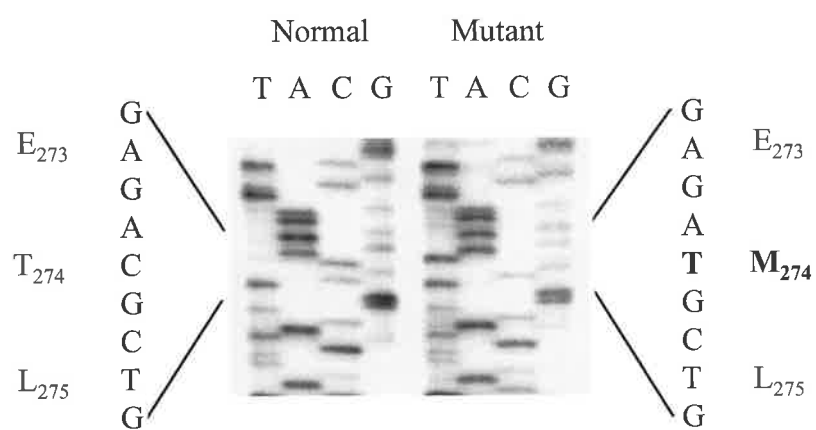
b) Sequence analysis of exon 4 ASA PCR products. Sequences are presented from an individual who was homozygous for the exon 4 SSCP change (mutant) and a control (normal). The deduced nucleotide and amino acid sequences are shown and altered sequences are indicated in bold. The loading order of the sequencing termination reactions are shown above the gel.

c) Filters 1 and 2 are duplicates containing exon 4 PCR products, probed with the normal (T274) and mutant (M274) ASOs respectively. Lanes 1 and 3 are from individuals who were heterozygous for the exon 4 SSCP change, lanes 2 and 4 are homozygotes for the SSCP alteration and lanes 5 to 8 contain PCR products from normal controls.

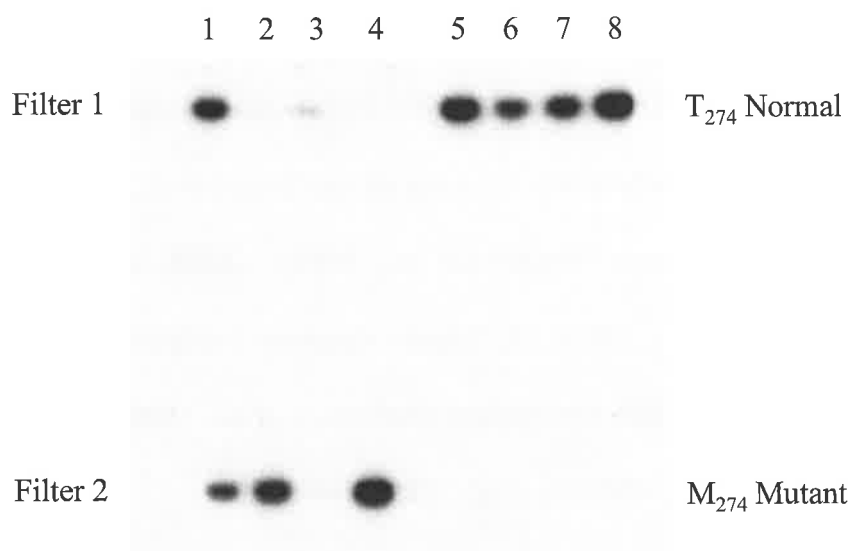
a



b



c



```

ARSA  --SFAERSGRGPFPGDSLMELDAAVGTLMTAIGDLGLLEETLVIFTADNGP-----
ARSB  PYDFIQDKNRHHYAGMVSLMDEAVGNVTAALKSSGLWNNTVFIFSTDNGGQ-----
STS   --DFAGKSQHGVDGAVEEMDWSVGQILNLLDELRLANDTLIYFTSDQGAHVEEV
SU1   --DFSCTSRGRYGDNLLEMHDAVQKIVDKLEENNISENTIIFFISDHGPHREYC
SU2   --DFSCTSRGRYGDNLREMDQAIEQIVTTLVDNDIDDNTVIFFTSDHGPHREYC
E.coli ----GSSPARTSYGDCMVEMNDVFANLYKTLEKNGQLDNTLIVFTSDNGPEAE--
Gal6S --PFLGTSQRGRYGDVREIDDSIGKILELLQDLHVADNTFVFFFTSDNGAAL---
2S    PVDFQ-RKIRQSYFASVSYLDTQVGRLLSALDDLQLANSTIIAFTSDHG-----
Glu6S -----QTLLSVDDLVEKLVKRLEFTGELNNTYIFYTSDNGYHT---
Cre   -----EVAGPVDELIEQVVKTLDEAGVLDNTYIIYSADNGYHV---
      ..      .      .      .      .      .      .      .      .
                                     . . *
                                     ↑

```

Figure 3.4 Conservation of the T274 residue among the sulphatases

Comparison of a portion of the amino acid sequence of ASA with the sequences of 9 other sulphatases near ASA T274. Positions where identical "*" and conservative "." amino acid substitutions occur in the ten sequences are indicated. The sequences are; ARSB, human arylsulphatase B (Peters *et al.*, 1990); STS, human steroid sulphatase (Yen *et al.*, 1987); SU1 and SU2, 2 arylsulphatases from sea urchin (Sasaki *et al.*, 1988; Yang *et al.*, 1989); *E. coli*, *E. coli* arylsulphatase (Murphy *et al.*, 1992); Gal6S, human galactosamine 6-sulphatase (Tomatsu *et al.*, 1991); 2S, human iduronate 2-sulphatase (Wilson *et al.*, 1990); Glu6S, human glucosamine 6-sulphatase (Robertson *et al.*, 1992); Cre, *Chlamydomonas reinhardtii* arylsulphatase (De Hostos *et al.*, 1989).

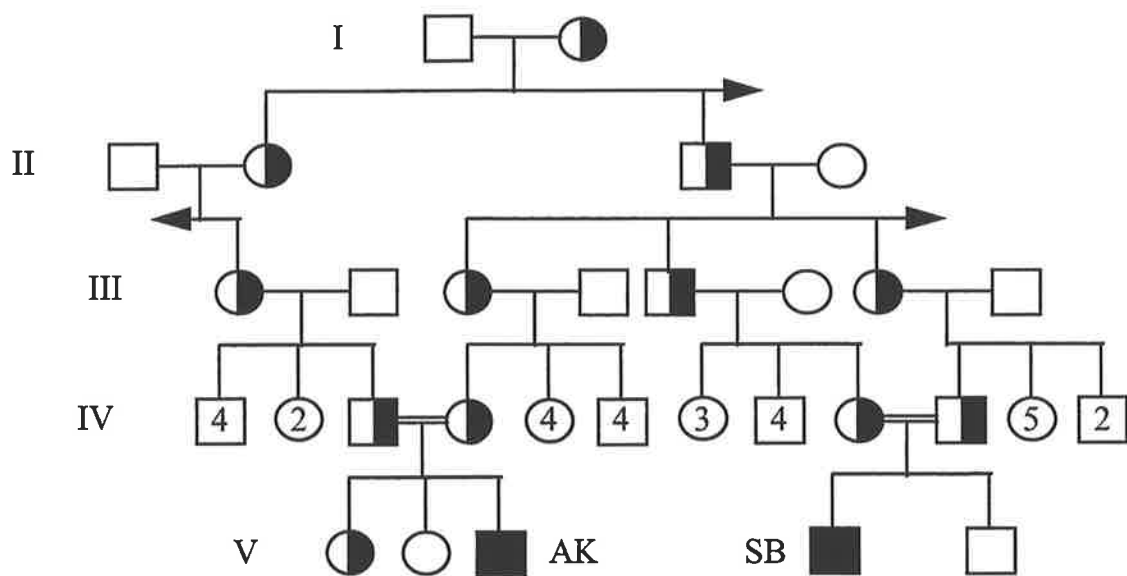


Figure 3.5 Inheritance of the T274M mutation

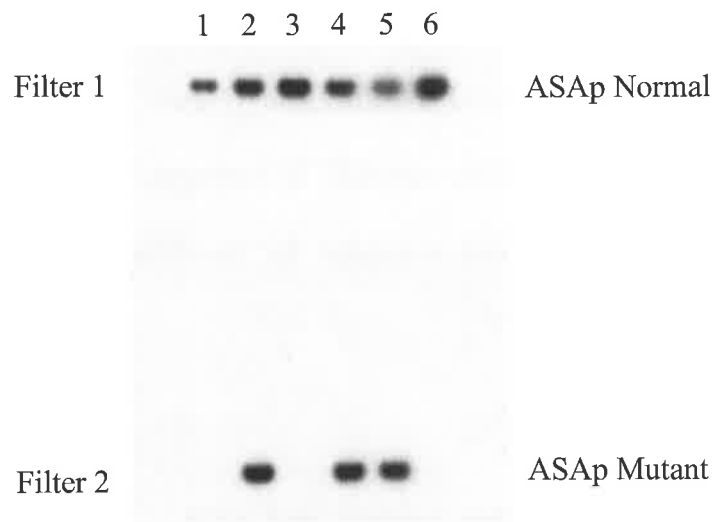
Five generations of a Lebanese Australian MLD family, with solid areas representing the mutant M274 allele and blank areas the normal T274 allele. AK and SB are MLD patients. Arrows indicate that extra individuals occur within a particular generation and numbers indicate numbers of normal children within the pedigree. Genotypes in generations I, II and III are inferred.

Figure 3.6 Screening for the T274M and pseudodeficiency allele

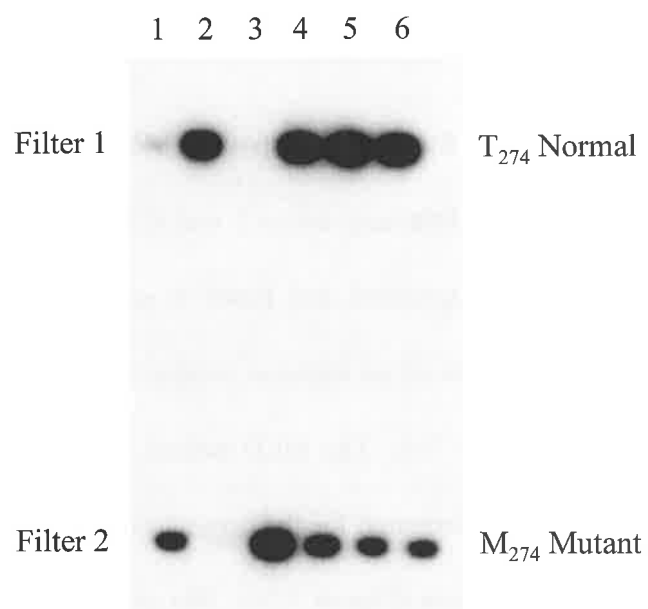
a) and b) show the analysis of duplicate filters with exon 8 (containing the ASA-PD polyadenylation defect) and exon 4 (containing the T274M mutation) PCR products respectively. Upper duplicates were hybridised with normal ASOs, and lower duplicates, mutant ASOs, corresponding to the ASA-polyadenylation defect (a) and T274M (b). Lanes 1 and 3 are affected children, lane 2 is an unaffected child, lane 4 is the misdiagnosed prenatal sample and lanes 5 and 6 are the parents.

c). Representation of the results on a pedigree. Individuals 1-6 correspond to the lane labels in a) and b). Solid areas represent the M274 mutation, hatched areas the ASA-polyadenylation defect, blank areas are normal alleles.

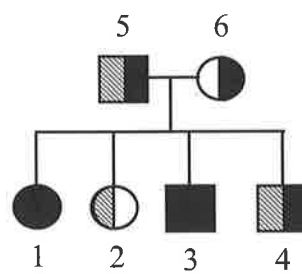
a



b



c



expected the affected children in the pedigree were T274M homozygotes and the parents and normal child were heterozygous for the allele. The misdiagnosed prenatal sample was a T274M/ASA-PD compound heterozygote (Figure 3.6c, individual 6). This genotype produces an extremely low, although in hindsight, non-pathogenic level of ASA activity (Penzein *et al.*, 1993) and accounted for the difficulty in unequivocally diagnosing the pregnancy.

3.3.2.b MLD and the ASA-pseudodeficiency allele

The identification of the ASA-PD allele is not necessarily diagnostic of ASA pseudodeficiency. Disease causing MLD mutations are equally as likely to occur on the ASA-PD allele as they are on the normal ASA allele (Gieselmann *et al.*, 1994).

SSCP analysis of the cDNA of patient MM identified a shift in the mobility of the PCR amplified product containing exons 3 and 4 (Figure 3.7a). The exon 3/4 PCR product from patient MM was sequenced and found to contain a single homozygous base change resulting in the substitution of an arginine residue for a proline at amino acid number 155 of the ASA cDNA (Figure 3.7b). The MLD patient, ASA-pseudodeficient and normal control populations (n=50) were screened for the presence of the P155R mutation. Only patient MM was found to carry the allele (Figure 3.7c). No other novel alterations were identified in an SSCP screen of either the genomic or cDNA copies of the ASA gene of patient MM.

MM was the only patient of Lebanese decent not to carry the T274M mutation. This was not surprising since the clinical presentation of the patient was distinct from that of the T274M homozygotes. The P155 residue was not well conserved (results not shown) among related sulphatase enzymes and the allele was not identified in any normal controls, the role

that the variant played in the presentation of MM was unclear, particularly in view of the co-inheritance of the ASA-PD allele with the P155R sequence variant.

Patient PG was found by SSCP analysis to be homozygous for an alteration in the exon 5 genomic PCR product (Figure 3.8a). Sequence analysis of the exon and its adjacent intron boundaries revealed a G to T transversion affecting the last nucleotide of exon 5 (Figure 3.8b). The MLD patient and pseudodeficient populations as well as fifty control individuals were screened for the G325C mutation and no other carriers were identified (data not shown). The parents and sibling of patient PG were confirmed as heterozygotes for the allele (Figure 3.8c).

The G325C variant not only caused an amino acid substitution (cystine for glycine at position 325) but had the potential to affect the splicing of the ASA mRNA since the mutation altered a nucleotide which formed part of the conserved nucleotide sequence in the splice donor site of the exon. In a review of eukaryotic RNA splice donor sequences by Shapiro and Senapathy (1987), approximately 80% of all mammalian exons were found to end with a G. Mutations affecting this nucleotide (like G325C) in general, resulted in the deletion, from the respective mRNAs of the exon in which the mutation occurred. The possibility that the G325S mutation affected the splicing of ASA was supported by the observation that amplification of exon 5/6 from the cDNA of patient PG produced three distinct bands, with the additional products being smaller than the original or wild type bands produced from all other MLD patients and controls.

ASO probes detecting sequences in exons 5, 6 and 7 of the ASA gene were used to analyse the three cDNA PCR products obtained from the amplification of exon 5/6 in patient PG. The three bands were difficult to analyse separately within a single track, consequently each of the three amplification products were isolated from an agarose gel as described in section 2.2.13.a. and electrophoresed in different lanes of a subsequent gel. (in triplicate),

Figure 3.7 Analysis of the P155R sequence variant

a) SSCP analysis of exon 3/4 cDNA PCR products. Lane 1 is a non-denatured control, lane 2 is patient MM and lanes 3 and 4 normal controls.

b) Automated sequence analysis of the exon 3/4 PCR product from patient MM. The mutant and normal gene sequences are presented above the figure.

c) Duplicate filters 1 and 2 contain exon 3/4 cDNA PCR products. Duplicates were hybridised with the P155 and R155 ASOs respectively. Tracks 1-8 and 10-12 contain amplification products from controls individuals, track 9 is patient MM.

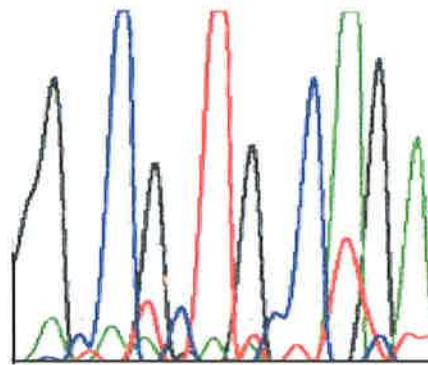
a



Normal: G C C C C T G C C A G A

Mutant: G C C G C T G C C A G A

b



c



Figure 3.8 Analysis of the G325C sequence variant

a) SSCP analysis of exon 5 genomic PCR products. Lanes 1 and 2 are normal controls, lane 3 is from patient PG.

b) Sequence analysis of exon 5 from control (normal) and patient PG (mutant) samples. The inferred nucleotide and amino acid sequences are given adjacent to each sequence. Altered amino acids and nucleotides are in bold. The order of the loaded sequencing termination reactions are shown above the gel for the mutant and normal tracks. Exonic and intronic sequences are represented in upper and lower case letters respectively.

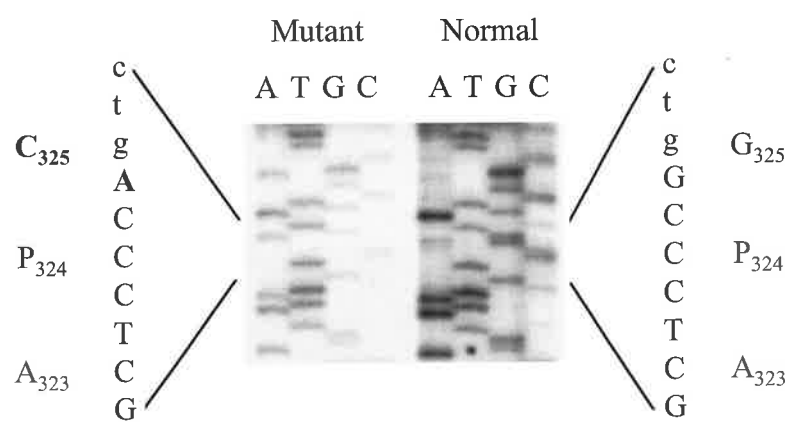
c) Filters 1 and 2 are duplicates containing exon 5 genomic PCR products, hybridised with the normal and mutant G325C ASOs respectively. Tracks 5-11 are normal controls, tracks 2-4 are the mother, father and sib of PG. Track 1 is patient PG.

a

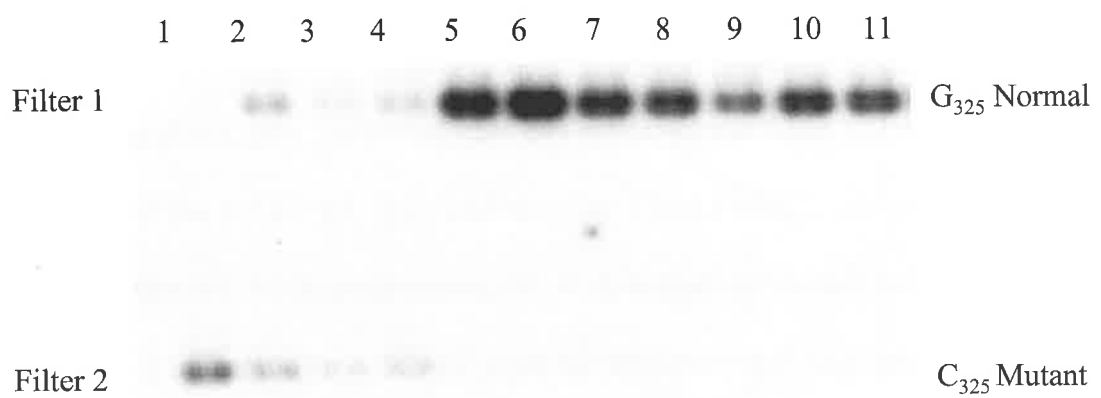
1 2 3



b



c



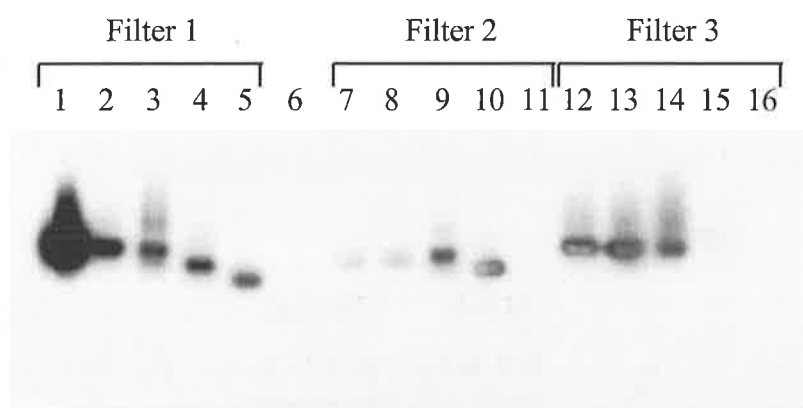


Figure 3.9 ASO analysis of patient PG exon 5/6 PCR products

Filters 1, 2 and 3 are triplicates and were hybridised with ASOs specific to exons 6, 5 and 7 respectively. Lanes 1 and 2 on each filter (1,2; 7,8; 12,13) are normal controls. The remaining lanes contain the largest (3; 9; 14) intermediate (4; 10; 15) and smallest (5; 11; 16) of the PCR amplified fragments from the exon 5/6 cDNA amplification from patient PG.

transferred to nylon membranes and probed with the exon specific ASOs. The results of this analysis appear in Figure 3.9. Filter 1 was hybridised with an exon 6 specific probe and all bands were found to contain this sequence. Filters 2 and 3 were probed with exon 5 and 7 specific probes respectively. The smallest of the PCR products was found to carry a deletion of exons 5 and 7 and the intermediate product a deletion of only exon 5. This finding was confirmed by sequencing the exon/exon boundaries of each PCR product (results not shown).

It was serendipitous that the forward and reverse primers used to amplify exons 5 and 6 of the ASA cDNA were designed to extend from exon 4 in the forward direction and from exon 8 in the reverse direction (owing to the small size of exon 7), this enabled the detection of all three splicing products from patient PG, purely by chance.

It is reasonable to assume that the ASA protein transcribed from mRNAs lacking entire exons would be unstable or non-functional, particularly considering that the deletion of exon 5 causes a shift in the reading frame of the protein. It is difficult and perhaps too speculative to estimate from PCR amplifications the relative amounts of the ASA mRNA species in patient PG which lack the exon 5 and 7 sequences, but roughly a quarter of the mRNAs seem to be correctly spliced (results not shown). As the G325C mutation was found in combination with the ASA-PD allele, which reduces the total amount of ASA mRNA, patient PG must produce only a very small amount of normally spliced message.

Even though the G325 residue is conserved in all but one of the related sulphatase enzymes in Figure 3.2, the presence of the G325C sequence variant in the small proportion of correctly spliced mRNA in patient PG is conceivably inconsequential to the overall ASA activity measured in the patient, compared with the effect that the G325C and ASA-PD alterations have on the production of normal ASA mRNA.

Figure 3.10 Identification and characterisation of the D169N sequence variant

a) SSCP analysis of exon 3 genomic PCR products. Lane 5 contains DNA from patient WT, lanes 1 to 4 are control individuals.

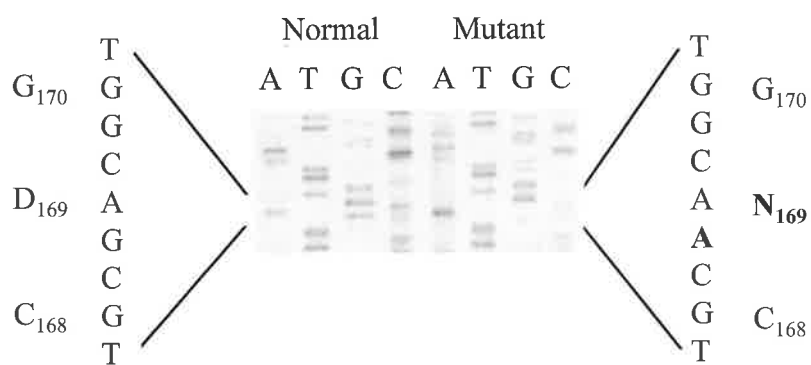
b) Sequence analysis of altered (mutant) and control (normal) PCR products. Inferred nucleotide and amino acid sequences are given adjacent to each sequence. The loading order of sequencing reactions are given above the figure. Altered nucleotides and amino acids are in bold.

c) Filters 1 and 2 are duplicates containing exon 3 PCR products hybridised with the normal and mutant D169N ASOs respectively. Lanes 1, 2 and 5 are normal controls, lanes 3 and 6 are heterozygous carriers of D169N and lane 4 is patient WT.

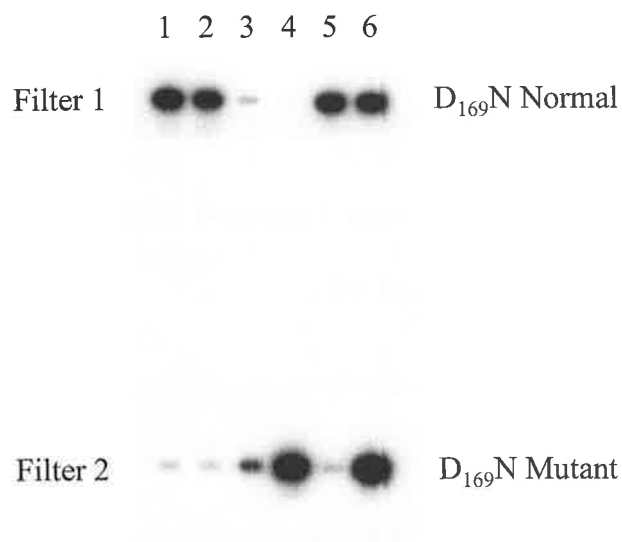
a



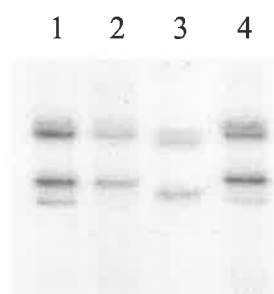
b



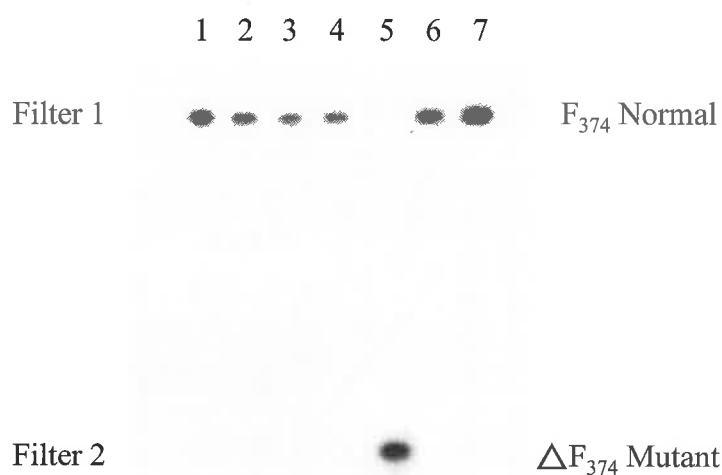
c



a



b



3.11 Characterisation of a deletion in exon 7 of the ASA gene

a) SSCP analysis of exon 7 genomic PCR products. Lanes 1, 2 and 4 are control individuals and lane 3 is patient WT.

b) Filters 1 and 2 are duplicates containing exon 7 genomic PCR products which were hybridised with normal (F_{374}) and mutant (ΔF_{374}) ASOs respectively. Lanes 1-4 and 6-7 are controls. Lane 5 contains PCR product from the genomic DNA of patient WT.

As was the case with patients MM and PG, patient WT (Table 3.1) was expected to carry an additional MLD mutation which would act to reduce the level of ASA activity expressed by the homozygous ASA-PD allele to a level sufficient to cause the onset MLD symptoms. SSCP analysis of this patient, however, revealed bands of altered mobility in exon 3 (Figure 3.10a) and exon 7 (Figure 3.11a). The patient appeared to be homozygous for both changes since the normal conformation products were absent and two aberrant products had appeared in each case. Sequence analysis of exon 3 and 7 PCR products from patient WT identified a G to A transition in exon 3 causing an aspartic acid to asparagine alteration at amino acid number 169 (Figure 3.10b) and an in-frame deletion of three base pairs in exon 7 which removed a phenylalanine residue from the polypeptide at position 374 (data not shown). ASO analysis revealed that WT was homozygous for both of these alterations (Figures 3.10c and 3.11b).

No carriers of the deletion were identified in any of the remaining MLD or pseudodeficiency patients (data not shown). Similarly, no carriers were identified in a screen of the normal population (n=33). In contrast, although no carriers of the D169N mutation were found in a similar population of controls, two pseudodeficiency patients were identified who carried the D169N mutation (data not shown). One pseudodeficiency patient was heterozygous and the other was homozygous for the allele. Both of these individuals had Polynesian ancestry while the ethnic origin of the original patient was not clearly established. In all cases however, the D169N mutation was inherited on the same allele as ASA-PD. The fact that this 'double mutant' allele was identified in the homozygous state in an individual not affected with MLD means that the allele is obviously non-pathogenic. The non-pathogenic nature of the D169N/pseudodeficiency allele implicates the amino acid 374 deletion as the pathogenic mutation in patient WT.

3.3.2.c Further novel ASA alleles

Four additional ASA sequence variants were identified in this study. Patient LP exhibited two genomic PCR products with altered mobility compared to normal controls. The first alteration was identified during analysis of exon 4 (Figure 3.12a) and the second was found in exon 5 (Figure 3.13a). Sequence analysis of the two alterations identified two C to T transitions, both of which caused the substitution of a cysteine residue for an arginine. The changes affected amino acids 244 (Figure 3.12b) and 288 (Figure 3.13b) in exons 4 and 5 respectively. ASO analysis of patient (n=29), pseudodeficient (n=21) and normal control populations failed to detect any further carriers of the alleles. A total of 79 normal controls were screened for the R244C and R288C alleles in an unsuccessful attempt to identify a normal carrier and hence distinguish which of the variants may have been pathogenic. Patient LP was found to be homozygous for the R244C mutation (Figure 3.12c) and heterozygous for the R288C mutation (Figure 3.13c). In addition, patient LP was shown to be heterozygous for the T391S sequence variant (Polten *et al.*, 1991).

SSCP analysis of genomic exon 7 and 8 PCR products detected two further conformational changes, which indicated the presence of sequence alterations in these products when compared to control bands. A heterozygous change was identified in patient KN and a homozygous change was found in patient SA in exons 8 (Figure 3.14a) and 7 (Figure 3.15a) respectively. Sequencing of the respective PCR products revealed a C to T transition in each exon. The alteration in patient KN produced a premature stop codon at residue number 486 (data not shown), which conceptually truncated the length of the ASA polypeptide by 21 amino acids. ASO analysis confirmed the presence of the mutation in patient KN but failed to identify any other carriers of the allele in the patient (n=29), pseudodeficient (n=21) or normal control populations (n=50) (Figure 3.15b).

A C to T transition in exon 7 (Figure 3.14a) of patient SA caused the substitution of a tryptophan residue for an arginine at amino acid number 390 of the ASA polypeptide (Figure 3.14b). SA presented with severe late-infantile MLD (see section 2.1.1) and since his Indian parents were first cousins, it was not surprising to find that SA was homozygous for the R390W allele. No other carriers were identified (Figure 3.14c).

A control individual also was identified with a band of altered conformation during the analysis of exon 7 PCR products in Figure 3.15a. This exon 7 PCR product was subsequently sequenced in Figure 3.15b and found to contain a heterozygous copy of the T391S sequence variant which had been previously identified by Polten *et al.* (1991).

Individuals 5 and 7 in Figure 3.15c were also found to be homozygous for the T391S allele (results not shown). This explains why neither the normal or mutant R390W ASOs hybridised to their respective PCR products. The presence of the T391S allele meant that their gene sequences were not homologous to either of the R390W allele specific oligonucleotides.

3.12 Analysis of the R244C sequence variant

a) SSCP analysis of exon 4 genomic PCR products. Lanes 1 and 3-5 are control individuals. Lane 2 contains amplified products from patient LP.

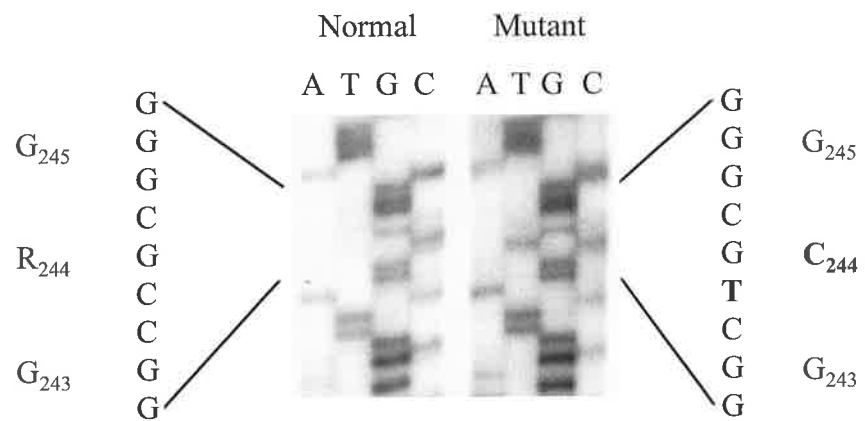
b) Sequencing of control (normal) and altered (mutant) exon 4 conformation products. The inferred nucleotide and amino acid sequences appear at the side of the figure. Altered bases are in bold. The loading order of the sequencing termination reactions appear at the top of the figure.

c) Hybridisation of the normal (filter 1) and mutant (filter 2) R244C ASOs to duplicate filters containing exon 4 amplified PCR products. Lanes 1 to 6 and 8 are controls, lane 7 contains the exon 4 PCR product from patient LP.

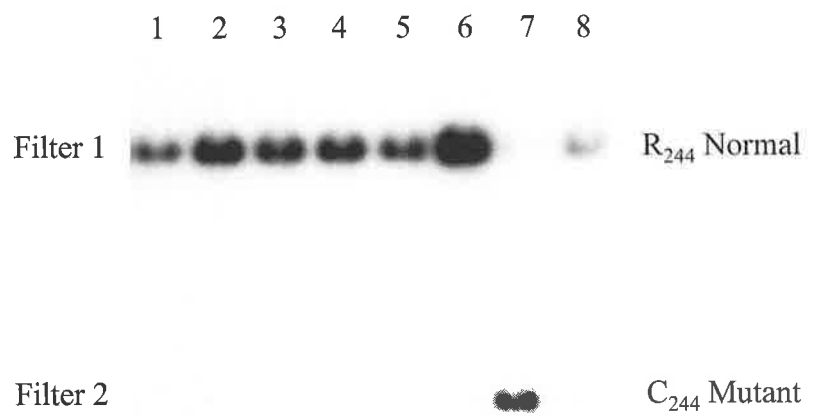
a



b



c



3.13 Characterisation of the R288C sequence variant

a) SSCP analysis of exon 5 genomic PCR products. Lanes 1 to 4 are control samples, lane 5 contains exon 5 genomic PCR product from patient LP.

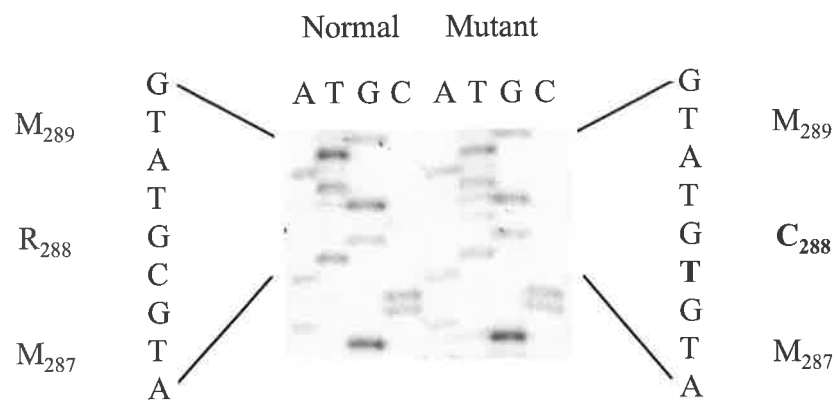
b) Sequence analysis of the altered and normal conformation products. The inferred nucleotide and amino acid sequence appears adjacent to the normal and mutant sequences. Altered amino acids and nucleotides are in bold. The loading order of the sequencing termination reactions appear above the figure.

c) Filters 1 and 2 are duplicates containing exon 5 genomic PCR products. The duplicates were hybridised with the normal and mutant R288C ASOs respectively. Lanes 1, 2 and 4-9 are controls, lane 3 contains exon 5 amplified genomic product from patient LP.

a



b



c

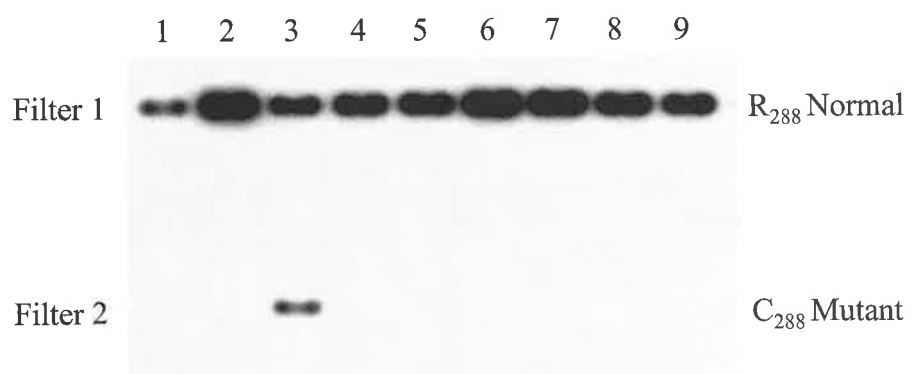


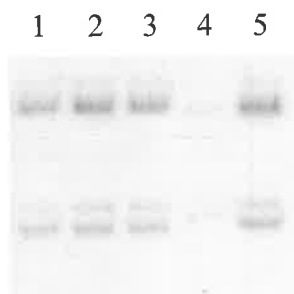
Figure 3.14 Identification and characterisation of the R390W sequence variant

a) SSCP analysis of exon 7 genomic PCR products. Lanes 1-3 and 5 are controls, lane 4 contains the amplified PCR product from patient KN.

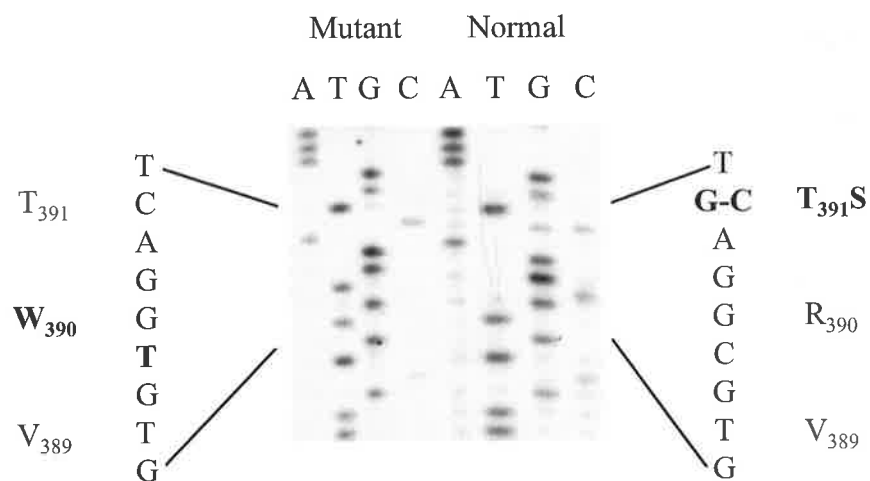
b) Sequence analysis of the altered conformation products in figure a, lanes 4 (patient KN) and 5 (normal control). The nucleotide and amino acid sequence of each individual appears adjacent to the figure. The loading order of the sequencing termination reactions appears above the figure. Altered nucleotides and amino acids are in bold.

c) Filters 1 and 2 are duplicates and contain exon 7 amplified PCR products. The filters were hybridised with the ASOs detecting the normal (R390) and mutant (W390) sequences respectively. Lanes 1 to 5 and 7 to 11 are control individuals, lane 6 contains amplified product from patient SA.

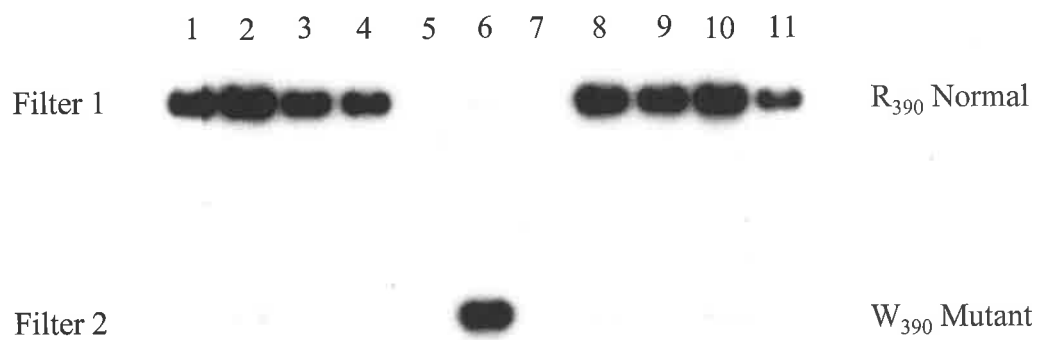
a



b



c



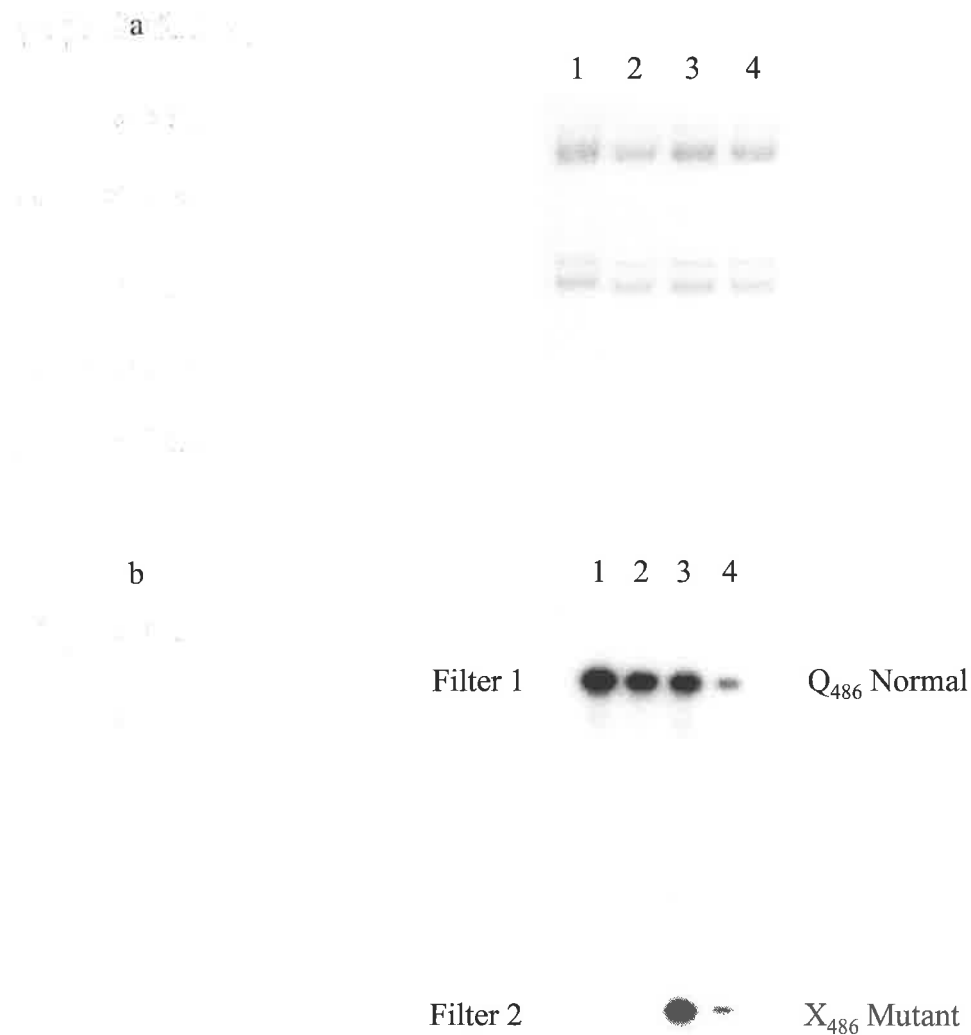


Figure 3.15 Premature termination of the ASA polypeptide

a) SSCP analysis of exon 8 genomic PCR products. Lanes 1 to 3 are normal controls, lane 4 contains exon 8 amplified genomic DNA from patient KN.

b) Hybridisation of duplicate filters 1 and 2 with the normal (Q486) and mutant (X486) ASOs respectively. Lanes 1 and 2 are control individuals, lanes 3 and 4 are from patient KN and his mother respectively.

3.4 CONCLUSIONS

3.4.1 Concluding Remarks

Analysis of the ASA gene for both previously identified and novel MLD alleles has allowed the characterisation of putative disease causing sequence variants in a large proportion of the MLD patient population. SSCP and sequence analysis was able to identify 23 out of a probable 26 mutant ASA alleles in the 13 patients in which no mutations were identified after the population had been screened for known alleles (Table 3.3). SSCP analysis was not expected to detect all sequence changes within the ASA gene. Some sequence alterations will conceivably not affect the conformation of a single-stranded PCR product and would therefore appear normal on an SSCP gel. Other pathogenic mutations may also have occurred in either the 5' or 3' untranslated regions of the gene or in the SAP I activator gene and would therefore not be detected by the SSCP screen of the ASA gene described in the thesis.

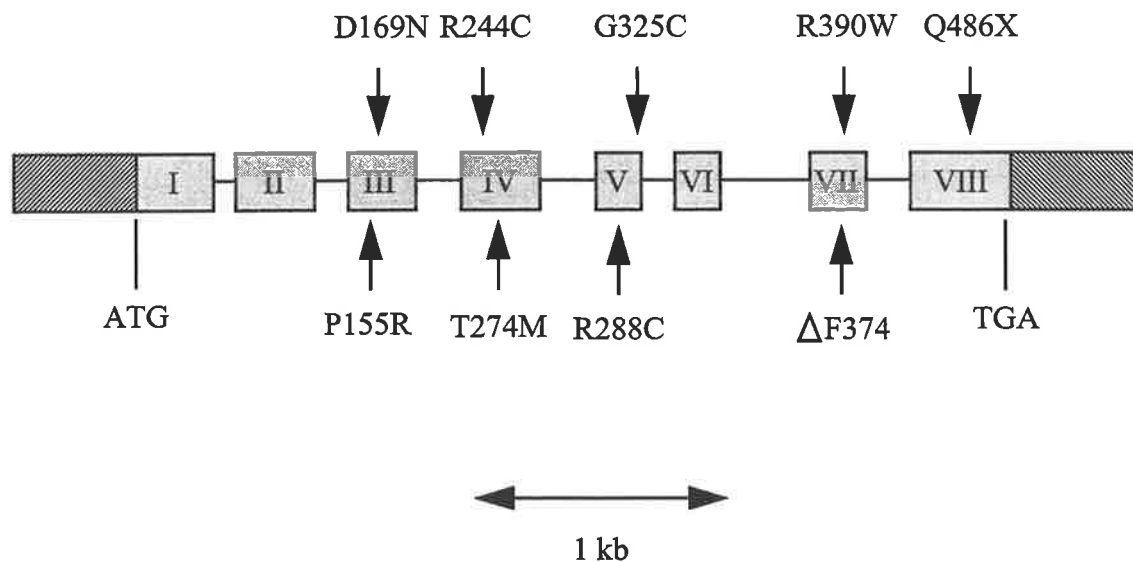
The novel MLD alleles identified in this study are summarised in Figure 3.16 and include seven missense mutations, a single nonsense mutation and a deletion.

The T274M mutation is clearly an important allele in the causation of MLD in the Australian Lebanese population, given its frequency in the patient population, the conservation of the residue among related sulphatase enzymes and the uniform presentation of T274M homozygotes with the severe late-infantile form of the disorder. Equally, the deletion of 21 amino acids from the ASA polypeptide due to the presence of the Q486X mutation would clearly be detrimental to the activity of the ASA protein by affecting either the catalytic potential or stability of the enzyme. Although a second allele has not been identified in patient KN, it would be expected to be a severe allele, that in combination with the Q486X mutation would account for the late-infantile presentation in this patient.

<u>Patient</u>	<u>ASAp</u>	<u>N350S</u>	<u>P155R</u>	<u>D169N</u>	<u>R244C</u>	<u>T274M</u>	<u>R288C</u>	<u>G325C</u>	<u>Del374</u>	<u>R390W</u>	<u>Q486X</u>	<u>T391S</u>
MM	++	++	++	--	--	--	--	--	--	--	--	--
LP	--	--	--	--	++	--	+-	--	--	--	--	+-
SA	--	--	--	--	--	--	--	--	--	++	--	--
PE	--	--	--	--	--	++	--	--	--	--	--	--
MG	--	--	--	--	--	++	--	--	--	--	--	--
CG	--	--	--	--	--	++	--	--	--	--	--	--
SG	--	--	--	--	--	++	--	--	--	--	--	--
SB	--	--	--	--	--	++	--	--	--	--	--	--
PG	++	++	--	--	--	--	--	++	--	--	--	--
AK	--	--	--	--	--	++	--	--	--	--	--	--
KN	--	--	--	--	--	--	--	--	--	--	+-	--
WT	++	++	--	++	--	--	--	--	++	--	--	--
GS	+-	+-	--	--	--	--	--	--	--	--	--	--

Table 3.3 The identification of novel ASA gene variants

The results of the screen of 13 MLD patients for novel ASA sequence variants, with “+” representing the presence of the mutant allele and “-” the normal allele. Included in this table are the previously characterised ASA-PD polyadenylation, N350S and T391S sequence variants to enable the presentation of complete patient genotypes.



Figures 3.16 Summary of novel ASA sequence variants identified by SSCP analysis

Schematic diagram of the ASA gene showing the novel ASA sequence variants identified as a result of SSCP and sequence analysis. Blocked areas represent the eight ASA exons which are numbered whilst hatched areas represent the 5' and 3' untranslated regions of the gene. ATG and TGA represent the initiation and termination codons of the gene. Arrows indicate the relative position of each sequence variant in the ASA gene.

Although, as discussed, some of the novel sequence variants identified in this study are clearly pathogenic, the pathogenicity of the remaining sequence variants remains a matter of question. This is particularly relevant in view of the lack of conservation of the altered residues among related sulphatase enzymes (data not shown), the inability to detect any of the alleles in the normal population and the fact that in some cases, more than one variant was identified within an allele. These complex alleles included those where novel ASA sequence variants were found in combination with ASA-PD.

There seem to be two plausible explanations for the presence of multiple variants within these complex ASA alleles. Either there is a single pathogenic mutation present with other alterations being inconsequential in terms of the causation of disease or, in contrast, all mutations within these complex alleles may be important in additively reducing the activity of ASA to a pathogenic level. Elucidating the effect that the presence of these multiple variants in the complex MLD alleles have on the activity of the ASA enzyme will require the expression of these sequence variants and combinations of sequence variants in a recombinant *in vitro* assay system.

3.4.2 Mutation, Polymorphism or Sequence Variant ?

The question of whether the alterations identified within the ASA gene should be classified as mutations, polymorphisms or sequence variants is one of considerable confusion. In a classical genetic sense, any heritable alteration within a gene is classified as a mutation and if this mutation occurs in the population at a frequency which is above a certain level, it is also classified as being polymorphic. There is, however, considerable debate about what this frequency should be. In the field of human genetics, confusion has arisen from the use of mutation and polymorphism because the terms have come to imply alterations within genes which cause disease and those alterations which are benign, respectively. This 'convention'

implies nothing of the relative frequency with which disease causing or the benign alterations within a gene occur within the population.



For the purpose of this thesis the term mutation will only be used to describe changes within the ASA gene which are known to cause MLD. If the role played by an alteration in the ASA gene, in the causation of disease has not been established, then such alterations will be referred to simply as sequence variants. The only changes within the ASA gene that will be referred to as 'polymorphic' are the ASA-PD and T391S sequence variants. When used, the term 'polymorphic' is not intended to imply anything about the role that these two sequence variants may play in the causation of MLD it is used simply in reference to the high frequency with which these two sequence variants occur within the normal control populations.

Chapter 4

Redefining the Arylsulphatase A

Pseudodeficiency Allele

4.1 INTRODUCTION

4.1.1 Opening Remarks

The identification of the ASA-PD allele within the MLD patient population has provoked the need to re-evaluate the role that the allele may play in the presentation of disease, particularly in view of conflicting reports of the possible role that the N350S sequence variant may play in biochemically defining the allele.

The ASA-PD allele is clearly non-pathogenic when it occurs in isolation. This seems to be true whether it occurs in ASA-PD homozygotes or in individuals who are compound heterozygotes of ASA-PD and an MLD mutation. I hypothesise however, that under certain circumstances, the allele may still play an important part, in contributing to the phenotype of an MLD patient and that the allele may therefore, still be considered 'pathogenic'.

Three MLD patients, showing both the clinical and biochemical features of the disorder, were identified as ASA-PD homozygotes in this study. The role played by the ASA-PD allele in determining the clinical phenotype of these patients was unclear, but it seemed reasonable to suggest that 'other factors' could have acted to further lower the level of ASA activity produced by the ASA-PD allele, to a level which became pathogenic in these patients. The potential importance of the ASA-PD allele in the presentation of these patients was reinforced by the observation that two of the three patients did not present with the late-infantile form of the disease. This suggests, that, in these cases, MLD was not caused by non-functional or null ASA mutations. This poses the question, would the level of ASA activity in these patients be pathogenic without the presence of the ASA-PD allele ?

4.1.2 Specific Aim

This chapter presents the results of the analysis of the N350S and polyadenylation sequence variants which constitute the ASA-PD allele, and aims to ascertain the respective contribution of each these defects to the biochemical phenotype of ASA-PD. A ribonuclease protection assay was used to quantify the loss of the 2.1 kb ASA mRNA species in ASA-PD homozygotes. Studies on the expression of the N350S ASA cDNA construct and the targeting of the N350S ASA protein to the lysosome were performed using methods described in Chapter 5.

4.2 SPECIFIC MATERIALS AND METHODS

4.2.1 Specific Materials

4.2.1.a Enzymes

Ribonuclease A	Boehringer Mannheim, Germany
Ribonuclease T1	Boehringer Mannheim, Germany

4.2.1.b Radiochemicals

[α - ³² P] UTP (6000Ci/mmol)	NEN [®] DuPont, Boston, MA, USA
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4.2.1.c Kits and miscellaneous materials

Riboprobe [®] System-T7	Promega Corp, Madison, WI, USA
Lysate Ribonuclease Protection Kit	United States Biochemical Corp. Cleveland, Ohio, USA.

4.2.1.d Solutions and buffers

Lysis solution (ribonuclease protection)	4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% w/v sarkosyl
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Proteinase K working solution	40% (w/v) proteinase K, 300 mM NaCl, 30 mM sodium citrate
RNase working solution	50 mM Tris pH 7.5, 0.4 M NaCl, 10 mM EDTA, 0.5 U/ul RNase A, 0.025 U/ul RNase T1, 5% (v/v) glycerol

4.2.1.e Plasmid vectors

Ribonuclease protection assay, GAPDH control plasmid template	Dr Greg Goodall, Department of Human Immunology, Hanson Centre, Institute of Medical and Veterinary Science, Adelaide, South Australia.
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4.2.2 Specific Methods

4.2.2.a Cloning of the ASA protection assay probe sequence

The ASA PCR product which was amplified using primers Q486X (Appendix 3) and ON5 (Gieselmann *et al.*, 1991) was ligated into the pGEM[®]-T PCR cloning vector according to the manufacturers instructions (Promega Corp). Plasmid DNA was isolated from transformed cells and the inserts were sequenced, to allow the isolation of a clone which contained the PCR-generated insert in the reverse orientation (3'-5'). The ASA and GAPDH control plasmid constructs were digested with *Not* I and *Dde* I respectively before they were used as template for *in vitro* T7 transcription of the ASA and GAPDH antisense RNA protection assay probes.

4.2.2.b Probe production and purification

Antisense ASA and GAPDH RNA probes were synthesised in the presence of [α -³²P] UTP using the Promega Riboprobe[®] System-T7 kit with materials and methods as recommended and supplied by the manufacturer. No unlabelled or cold UTP was added to the synthesis reactions when transcribing the ASA antisense RNA probe however, when transcribing the GAPDH control antisense probe, the ratio of cold UTP to labelled UTP was

1000:1. The 20 ul synthesis reaction volume was increased to 100 ul with H₂O and the probe solution was subsequently spun through a Sephadex G-50 column to remove unincorporated nucleotides. Ribonuclease probes were labelled by nucleotide incorporation up to a specific activity of $>1 \times 10^6$ counts per minute (cpm)/ul of probe solution.

4.2.2.c Ribonuclease protection analysis

Total cell lysates were made from large, confluent T70 flasks of cultured skin fibroblasts. Cells were harvested as previously described and rinsed twice in standard saline (0.9% w/v NaCl) before the cell pellet was dissolved in 400 ul of lysis solution and stored at -20°C until required.

For each sample to be analysed, 1×10^6 cpm of the ASA probe and 1×10^4 cpm of the GAPDH control probe were mixed and diluted in 5 ul of lysis solution. The diluted probes was added to either 45 ul of total cell lysate or 3 ug of total RNA diluted to a volume of 45 ul with lysis solution. The hybridisation mixtures were heated to 90°C for 3 min before the antisense RNA probes were allowed to hybridise to target transcripts at 37°C overnight.

RNase working solution (500 ul) was added to each hybridisation, the mixture was briefly vortexed then incubated at 37°C for 60 min, after which 20 ul of 10% (w/v) SDS and 20 ul proteinase K working solution was added. The mixture was vortexed briefly before incubation for a further 30 min at 37°C. An equal volume (500 ul) of isopropanol was added to each hybridisation and mixed before centrifugation at 13000 g for 10 min at room temperature. The resulting pellet was air dried before resuspension in 6 ul of 10 x formamide load buffer. This solution was heat denatured at 90°C for 3 min then chilled on ice immediately prior to loading on a 6% (19:1) polyacrylamide, 7 M urea gel containing 1 x TBE as running buffer. Gels were typically run at 65 W constant power for approximately

120 min before the gels were blotted onto 3MM paper, vacuum dried and exposed to autoradiograph film overnight. The gels were subsequently exposed to a phosphorimaging cassette for quantification of the bands of interest.

4.2.2.d Analysis of RBNP results

Bands representing the protected 2.1 kb and 3.7 + 4.8 kb ASA mRNAs as well as the GAPDH control were quantified using the integral volume functions of Molecular Dynamics, ImageQuant version 3.3 as described by the supplier. In all cases except for the quantification of the 2.1 kb protected bands in pseudodeficient individuals, a line graph showing pixel volume across the band was plotted and a base line inserted to account for the measurable background in the track. The pixel volume of the curve above the baseline was measured to quantify the pixel volume of the band. When quantifying the volume of the protected 2.1 kb mRNA in pseudodeficient individuals, it was not possible to plot the bands pixel volume as a line graph because the bands were simply too faint. In these cases the protected band (and only the band) was boxed and the pixel volume of the box was measured. A box of identical size was placed immediately above and below the protected band. The average pixel volume of these two controls was taken as the background pixel volume of the band. All raw pixel volume data appears in Appendix 5.

The 2.1 kb ASA mRNA protected fragment contained 29 incorporated UTPs and the 3.7 + 4.8 kb contained 43, the pixel volume of the larger protected fragments was therefore multiplied by 0.672 to account for the differences in label incorporation.

4.3 RESULTS AND DISCUSSION

4.3.1 The Effect of the N350S Sequence Variant on the *in vivo* Expression of ASA

An ASA expression construct encoding the N350S sequence variant was transfected into Chinese hamster ovary (CHO) cells. The activity of the construct was subsequently measured in mass cultures of G-418 resistant cells as described in Chapter 5. The expression of the mutant construct was compared to that of the normal ASA construct, to determine the effect that the presence of N350S had on the activity of the ASA enzyme. Figure 4.1 shows the relative level of expression measured in the N350S construct over repeated assays. The average level of expression was 57% ($\pm 15\%$) of normal (n=3).

The N350S sequence variant alters one of three glycosylated asparagine residues found in the normal ASA polypeptide. As discussed in chapter 1, phosphorylated mannose residues on the N-linked oligosaccharide side chains of lysosomal enzymes are specifically targeted by the mannose 6-phosphate receptors of the cell, to transport newly synthesised enzymes (such as ASA) to the lysosome. Any alteration to the pattern of glycosylation in the ASA polypeptide could conceivably affect the targeting of the protein to the lysosome and hence reduce the overall *in vivo* amount of ASA which is available for the degradation of sulfatide.

Organelles from CHO cell expression lines containing the normal and N350S ASA constructs were fractionated on Percoll[®]/sucrose density gradients (As described in Chapter 5), with a confluent flask (T75) of cells containing each construct being used for each targeting experiment. The density gradients were aliquoted into 21 fractions of approximately 1 ml. β -hexosaminidase (β -hex) was used to detect fractions containing lysosomes (Leaback and Walker, 1961; Gieselmann *et al.*, 1989).

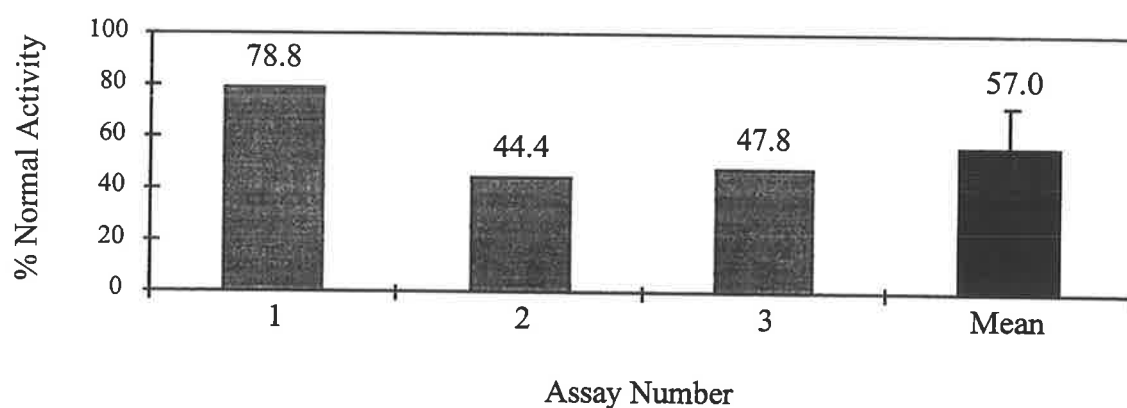


Figure 4.1 Expression of the N350S sequence variant

The ASA cDNA construct containing the N350S sequence variant was assayed in 3 replicate experiments (1 to 3). The measured level of expression is calculated as a percentage of that measured simultaneously in the normal expression construct. The right-hand bar of the graph represents the mean of the three experiments and the error bar represents one standard deviation from the mean.

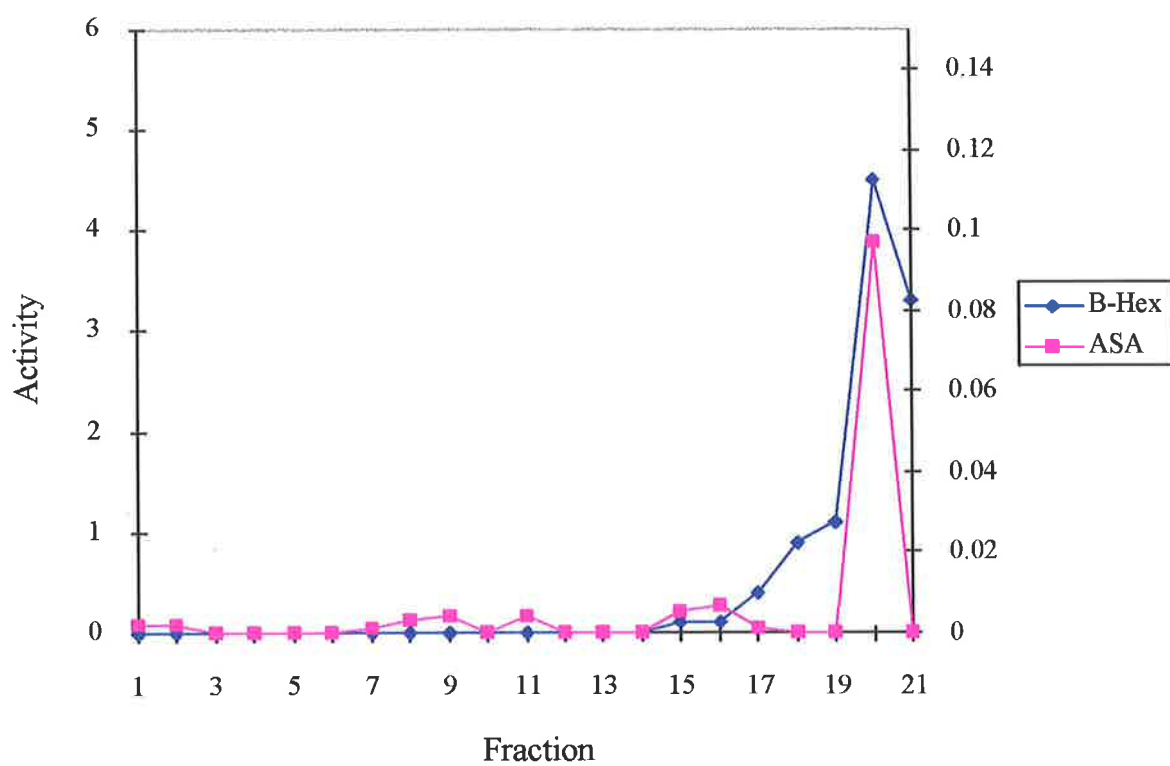
In fractions of organelles, from cells containing both the normal and N350S expression constructs, the highest activity of β -hex was identified in the 20th most dense fraction. The fractions from both gradients were also assayed for ASA activity (Figure 4.2). As was the case with the assay of β -hex, the peak level of ASA activity in cells expressing the normal construct (Figure 4.2a) was identified in the 20th fraction, indicating that the measurable ASA activity in the gradient was confined to the lysosome. In contrast, a substantial proportion of the ASA enzyme containing N350S was found in non-lysosomal fractions (Figure 4.2b). From this experiment it can be estimated that 55% of the N350S ASA enzyme is incorrectly targeted to the lysosome and is therefore unavailable for the *in vivo* hydrolysis of cerebroside sulphate.

The results presented here strongly suggest that the presence of the N350S amino acid substitution within the ASA protein, significantly affects the *in vivo* activity of the ASA enzyme, by not only reducing the catalytic activity of the enzyme but also by affecting the targeting of the synthesised N350S protein to the lysosome. The combined effect of the reduction in enzyme activity and the mistargeting of a proportion of the synthesised enzyme, is estimated to reduce the level of *lysosomal* ASA activity to about 25% of normal. The presence of N350S must therefore substantially contribute to the biochemical phenotype of the ASA-PD allele.

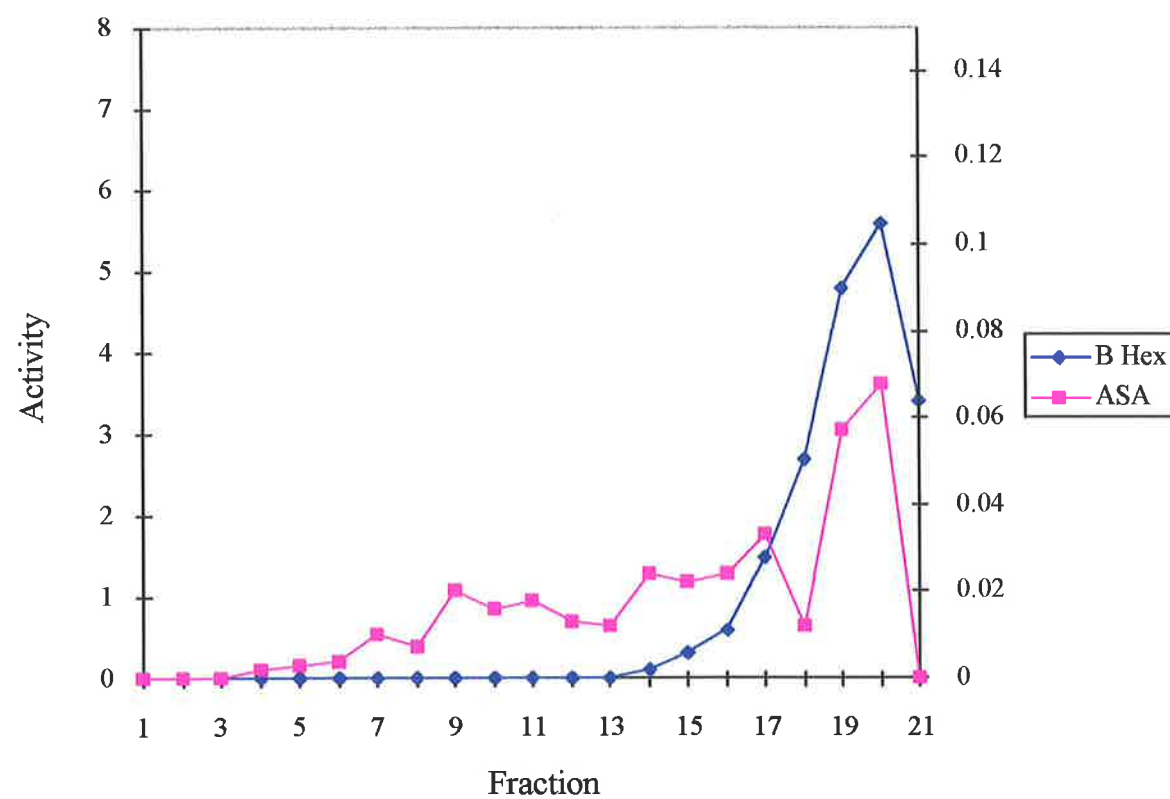
Figure 4.2 Density fractionation of cellular organelles

Analysis of Percoll[®]/sucrose gradient fractions from CHO cell lines expressing the normal (a) and N350S (b) ASA expression constructs. On both graphs, units on the left Y axis represent β -hex activity in nmol/min/mg of cellular protein. Units on the right Y axis are A_{515} values, measured from the assay of ASA with the artificial substrate, p-nitrocatechol-sulphate.

a



b



4.3.2 ASA mRNA Levels and the ASA-Pseudodeficiency Polyadenylation Defect

4.3.2.a Use of ASA polyadenylation sites in normal controls and ASA-PD homozygotes

A ribonuclease protection (RBNP) assay was used to measure the proportional loss of ASA mRNA, associated with the presence of the ASA-PD polyadenylation defect, as outlined in Figure 4.3. The gene encoding the glycolytic enzyme, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control in the RBNP assays. There are approximately 100 copies of the GAPDH gene in the human genome (Tso *et al.*, 1985) and like ASA, GAPDH is expressed as a house-keeping gene. The GAPDH mRNA transcript was used as an internal standard to which the abundance of the ASA mRNA transcripts could be compared. The degree of hybridisation between the GAPDH antisense RNA probe and its homologous mRNA, also allowed for compensation between tracks on the RBNP gels which contained different amounts of RNA. The ratio of the ASA mRNA transcripts to that of GAPDH was considered as constant between different cell lines and different tracks on the RBNP gels, regardless of the total amount of RNA that had been loaded (Lagardo *et al.*, 1994).

The low level of cellular ASA transcription made the detection of ASA mRNA using RBNP assays extremely difficult when using the standard protocols outlined by the supplier (USB). It was necessary to label the ASA antisense RNA probe to a specific activity of greater than 10^6 cpm/ul of probe solution, to enable the detection of the ASA transcripts. The higher level of transcription of GAPDH enabled the detection of the control transcripts with a probe which had a specific activity 1000 times lower than that used to detect ASA.

The antisense RNA probes were used to measure the abundance of the ASA and GAPDH mRNA transcripts in normal and pseudodeficient individuals. The results of this analysis are presented in Figures 4.4 and 4.5 for the analysis of cell lysates and in Figure 4.6

where total RNA was used as the target for the hybridisation of the antisense RNA probes. The raw pixel volume data is presented in Appendix 5.

The 2.1 kb and combined 3.7 + 4.8 kb ASA mRNAs could be detected in total cell lysate, but the bands on the RBNP gels which corresponded to the respective protection products, were only just visible above the high level of background hybridisation in the gel (Figure 4.4a). It was hoped that a reduction in the amount of ASA probe in the hybridisation reactions, would produce similar protection products without the extensive background noise. It was reasoned that the amount of ASA probe added to the hybridisation mixtures was far in excess of that required to hybridise to all available ASA transcripts and that the excess probe was simply adding to the background level of hybridisation on the RBNP gels. The result of this alteration to the protocol was not significant, although the number of background counts within the tracks of the RBNP gel decreased, the intensity of the protected ASA bands seemed to be equally reduced (Figure 4.4b).

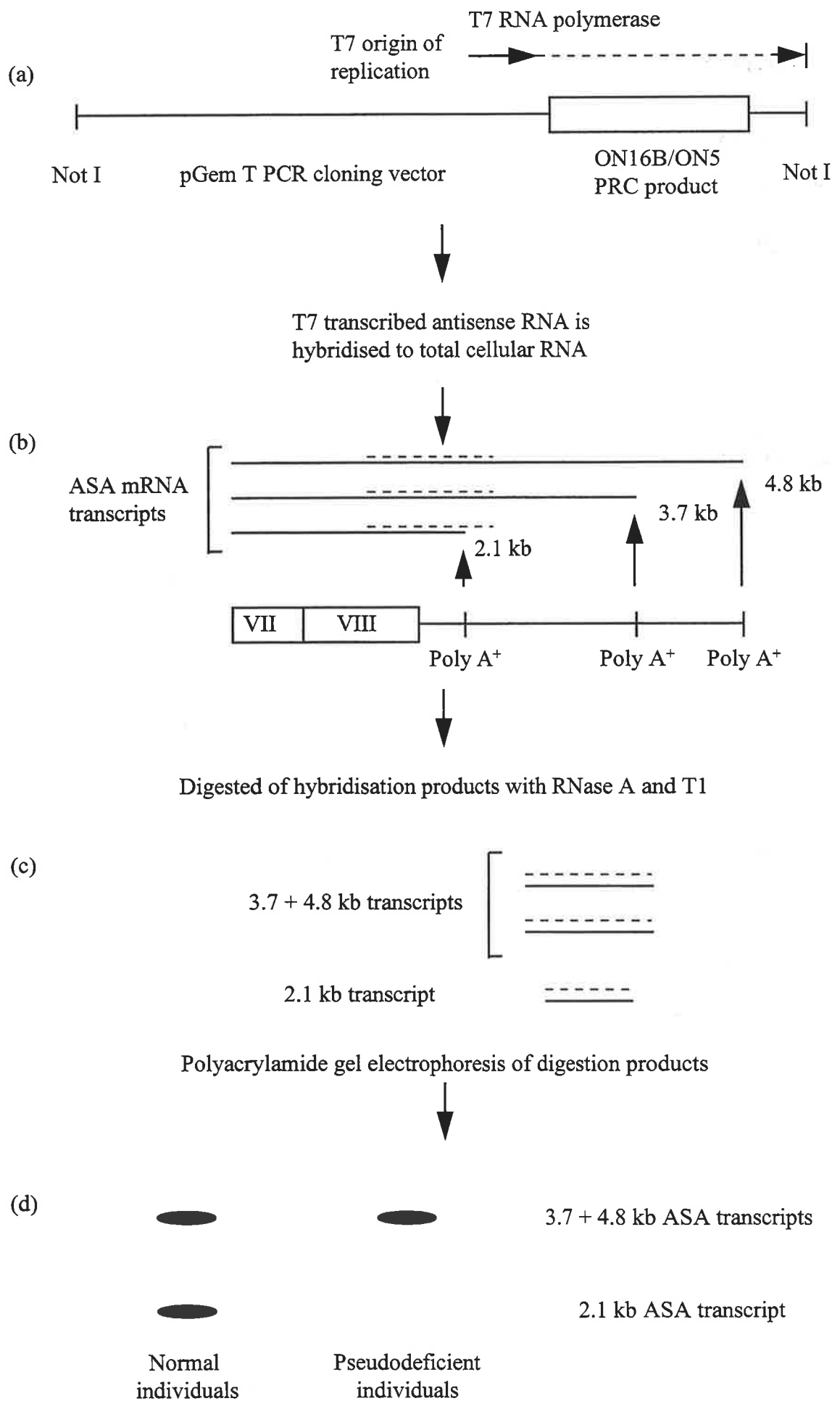
The alternative to the use of total cell lysate in the RBNP assays was the utilisation of total RNA as the template for the hybridisation of the RBNP antisense probes. The prior purification of the RNA did enable the amount of background hybridisation in the RBNP gels to be decreased as demonstrated in Figure 4.5.

Bands representing the 2.1 kb and 3.7 + 4.8 kb ASA mRNAs, as well as the GAPDH control were quantified on the cell lysate and total RNA protection assay gels. This analysis showed that in normal individuals (n=7) the 2.1 kb mRNA species accounted for an average of $70\% \pm 2.5\%$ of the total available ASA mRNA. The remaining 30% was made up by the two longer transcripts (Figure 4.6). Conversely, in individuals who were homozygous for the ASA-PD polyadenylation defect (n=5), the frequency of the major 2.1 kb transcript was reduced to almost undetectable levels. The 3.7 kb and 4.8 kb species, that in normal controls

Figure 4.3 Outline of ribonuclease protection assays

Linearised plasmid constructs containing ASA and GAPDH inserts downstream of a T7 RNA polymerase binding site, were used as templates for the synthesis of [$\alpha^{32}\text{P}$] UTP-labelled antisense RNA probes of known length.

- a) The ASA insert which spanned the 2.1 kb polyadenylation site was PCR amplified using the forward and reverse primers, ON16B and ON5. This amplified product was cloned into the pGem[®]T PCR cloning vector (Promega Corp.)
- b) Antisense ASA and GAPDH probes were hybridised to target RNA sequences in solutions containing total RNA or cell lysate preparations. The ASA antisense RNA probe hybridises to each of the polyadenylated ASA mRNAs (2.1, 3.7 and 4.8 kb).
- c) RNA/RNA hybrids are protected from RNase digestion, while all remaining single stranded RNA is degraded.
- d) The double-stranded RNA products remaining after RNase digestion were precipitated and pelleted before resuspension and size fractionation by polyacrylamide gel electrophoresis. Gels were exposed to autoradiographic film and to phosphorimaging cassettes for quantification of the protected ASA and GAPDH bands. The theoretical results of the analysis of the ASA mRNAs are presented. The 2.1 kb fragment is differentiated from the longer hybridisation products since the 2.1 kb ASA mRNA does not have homologous sequences to the entire length of the ASA probe. Both protection products should be seen in normal individuals whilst the 2.1 kb ASA mRNA is hypothesised as being absent in pseudodeficient individuals.



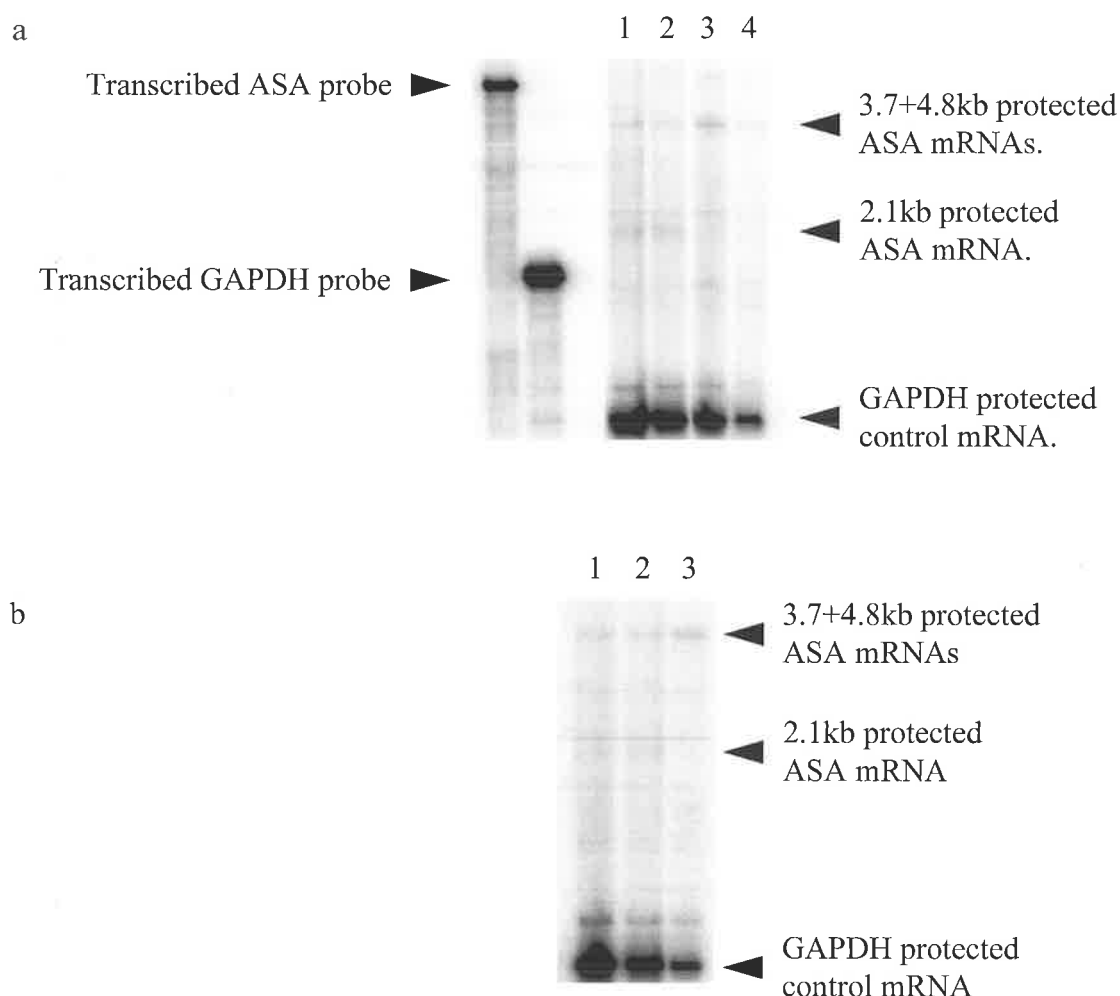


Figure 4.4 Ribonuclease protection analysis of cell lysates

a) The first two unmarked tracks contain the ASA and GAPDH *in vitro* transcription products which are 300 and 164 residues in length respectively. The third unmarked lane is blank. Lanes marked 1 and 2 contain RBNP products from the hybridisation of the ASA and GAPDH antisense probes to total cell lysates from normal individuals and tracks 3 and 4 are from lysates derived from pseudodeficiency homozygotes.

b) Lanes 1 and 2 are RBNP products derived from the cell lysates of normal individuals and lane three is from a pseudodeficient individual.

In figures a and b, the protected GAPDH product is 120 nucleotides in length. The 2.1 kb and 3.7 + 4.8 kb ASA protected products are 192 and 264 nucleotides in length, respectively.

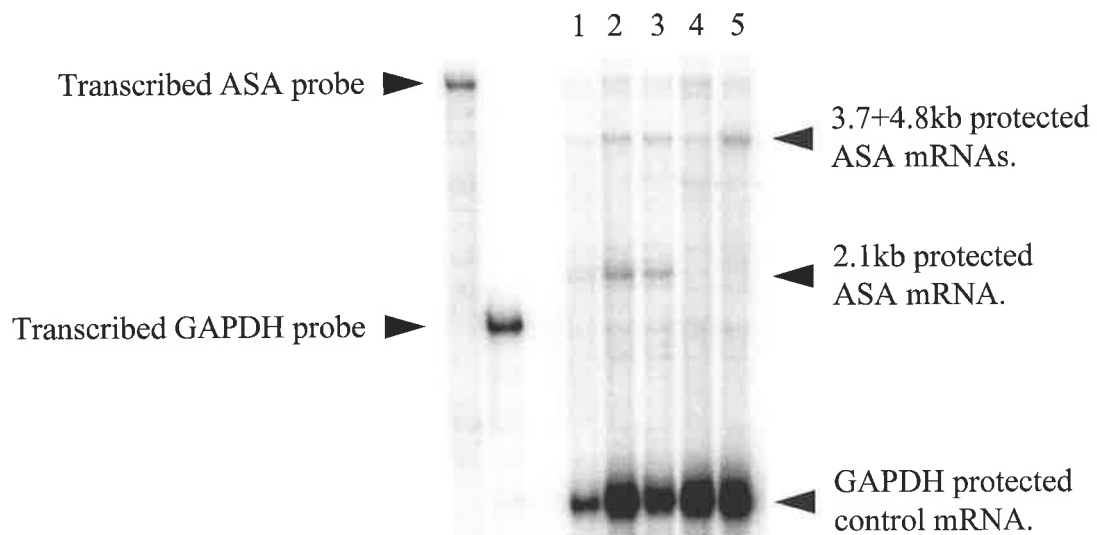


Figure 4.5 Ribonuclease protection analysis of total RNA

The first two tracks (unmarked) contain the ASA and GAPDH *in vitro* transcription products. The ASA and GAPDH probes are 300 and 164 residues in length respectively. The third unmarked track is blank. Lanes 1 to 5 contain the products of the ribonuclease protection assays, with the ASA and GAPDH antisense probes hybridised to total RNA.

Tracks 1-3 contain total RNA derived from normal individuals, whilst tracks 4 and 5 contain RNA from individuals who are homozygous for the ASA-PD polyadenylation defect. The transcribed GAPDH control probe protects a 120 nucleotide fragment of the GAPDH mRNA. The ASA RBNP probe protects 192 and 264 nucleotides of the 2.1 kb and 3.7 + 4.8 kb ASA mRNA transcripts respectively.

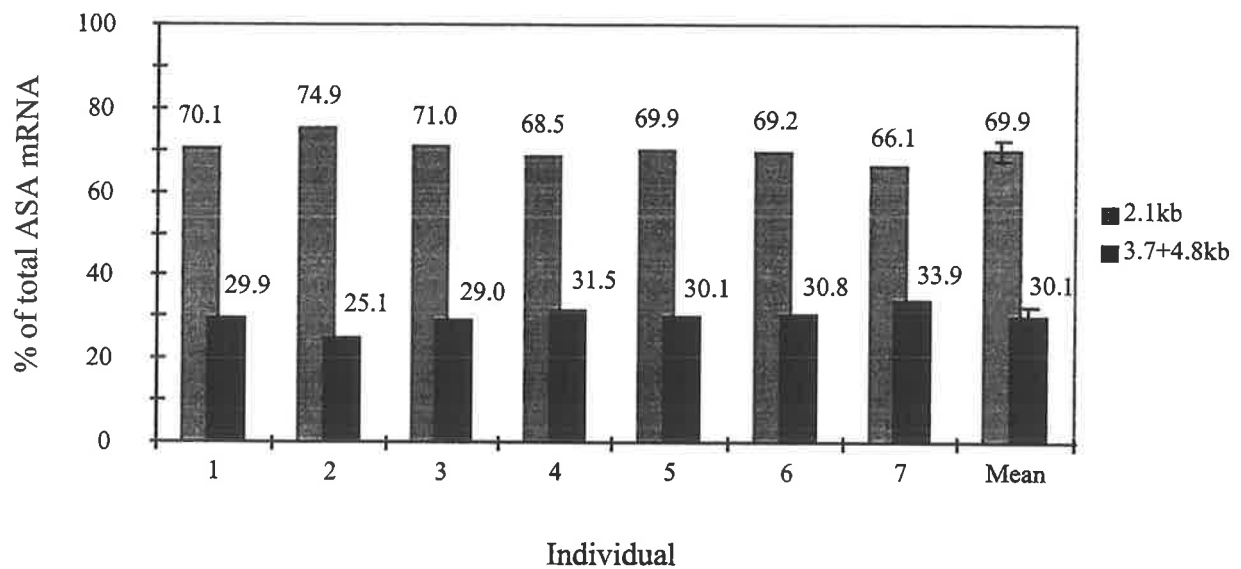


Figure 4.6 Polyadenylation of ASA mRNA in normal individuals

Use of the ASA polyadenylation sites in seven normal individuals. Columns 1-7 represent the percentage of total ASA mRNA contributed by the 2.1 kb and combined 3.7 and 4.8 kb polyadenylation sites in each normal control.

Individuals 1 to 7 represent individuals shown in tracks 1, 2 and 3 of Figure 4.5, tracks 1 and 2 of Figure 4.4a and tracks 1 and 2 of Figure 4.4b. Column eight presents the mean contribution of the 2.1 kb and 3.7 + 4.8 kb ASA mRNAs to total ASA mRNA in normal controls. The error bars represent 1 standard deviation from the mean.

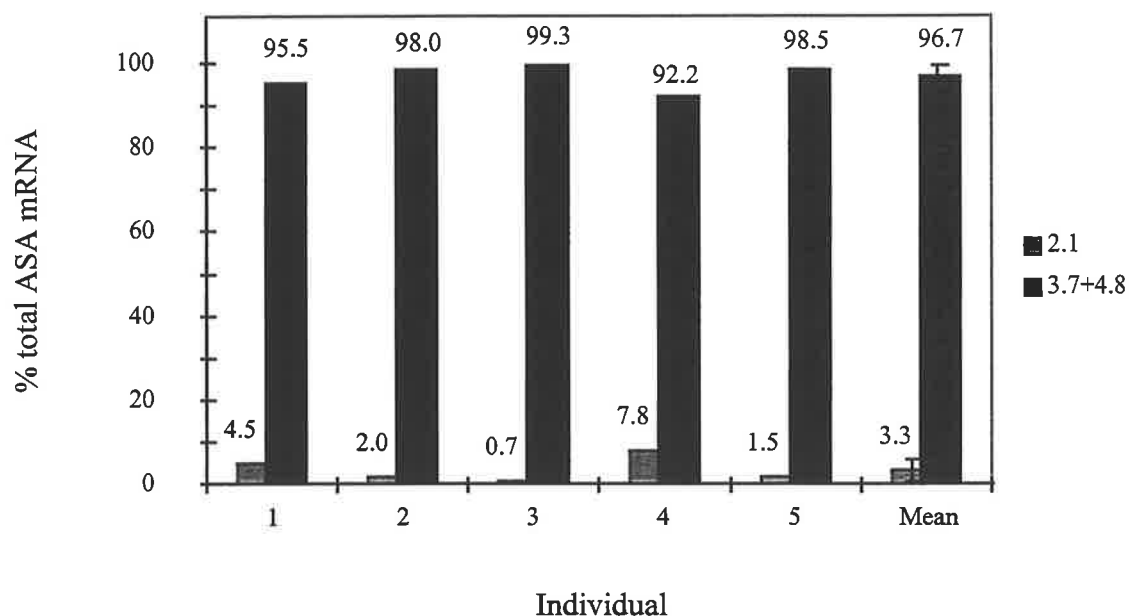


Figure 4.7 Polyadenylation of ASA mRNA in pseudodeficient individuals

Use of the ASA polyadenylation sites in five ASA-PD homozygotes. Columns 1-5 represent the percentage of total ASA mRNA contributed by the 2.1 kb and combined 3.7 and 4.8 kb polyadenylation sites in each pseudodeficient individual.

Columns 1 to 5 represent, in order, the pseudodeficient individuals shown in, tracks 4 and 5 of Figure 4.5, tracks 3 and 4 of Figure 4.4a and track 3 of Figure 4.4b. Column six gives the mean level of use of the polyadenylation sites and the error bars represent one standard deviation from the mean.

represented only 30% of total ASA mRNA, contributed $96.3\% \pm 3.0\%$ of the total ASA mRNA in ASA-PD homozygotes (Figure 4.7).

The relative use of the 2.1 kb and the combined 3.7 + 4.8 kb polyadenylation sites was reasonable consistent between the seven normal and five pseudodeficient individuals used in this study. This is despite of the degree of background hybridisation seen on the RBNP gels used to analyse the ASA and GAPDH transcripts from total cell lysates (Figure 4.4 a and b). It was thought that this degree of background hybridisation would bring a certain amount of variability to the pooled figures used to calculate the average contribution of each polyadenylation site to the total amount of ASA mRNA in each group.

4.3.2.b Relative use of the 3.7 + 4.8 kb polyadenylation signals

The question of whether the 3.7 kb and 4.8 kb polyadenylation sites are utilised with an increased frequency in pseudodeficient individuals is an important one. Any compensation for the loss of the 2.1 kb polyadenylation site by increased utilisation of the downstream termination signals would act to alter the deleterious biochemical effect of the ASA-PD polyadenylation defect and hence reduce its relative contribution to the reduction of ASA activity caused by the presence of the ASA-PD allele.

The ratio of the frequencies of the 2.1 kb and 3.7 + 4.8 kb ASA transcripts to that of GAPDH, was calculated for each of the normal and pseudodeficient individuals analysed in Figures 4.4a and 4.5. The RBNP reactions presented on these figures contained equal amounts of the ASA and GAPDH antisense probes and were hybridised to cell lysate and total RNA respectively, the individuals analysed in Figure 4.4b were excluded because less ASA probe was added to the hybridisation mixture in an attempt to reduce the background level of hybridisation. As expected, comparison of the frequency of the 2.1 kb ASA mRNA in normal and pseudodeficient individuals showed a marked reduction (98%-99%) in the amount of this

transcript in the presence of the ASA-PD polyadenylation variant. The ratio of GAPDH to the 2.1 kb mRNA was 0.0946 and 0.00144 for normal and ASA-PD individuals (Figure 4.8).

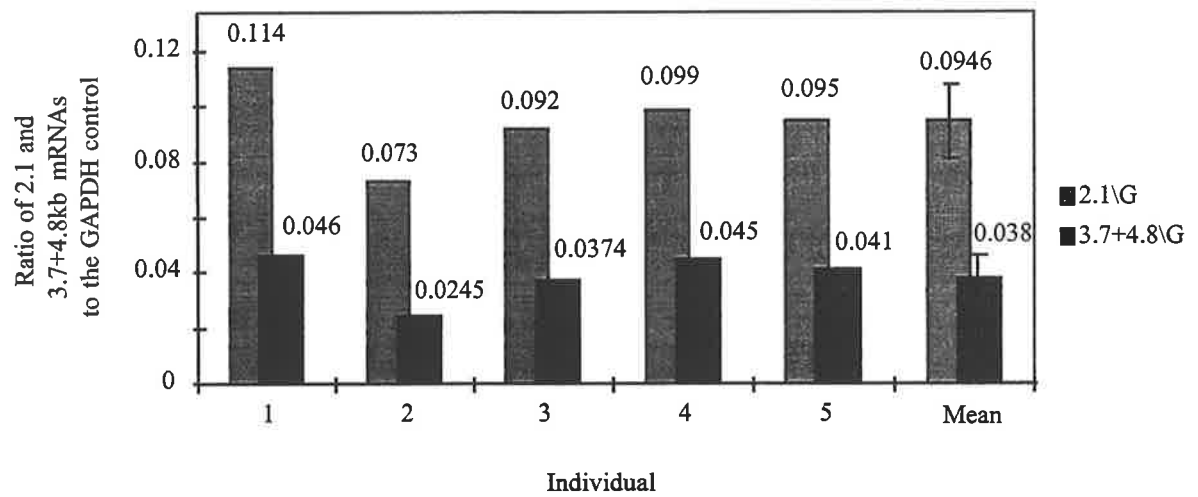
Of greatest interest, was the observation that there was no evidence to suggest any increased usage of the 3.7 kb and 4.8 kb ASA polyadenylation sites in pseudodeficient individuals (Figure 4.9). When comparing the use of the 3.7 and 4.8 kb polyadenylation signals, relative to the GAPDH control, in normal and pseudodeficient individuals, the average usage is almost identical (Figure 4.9). However, there was more variation, (0.039 ± 0.01 in pseudodeficient individuals as opposed to 0.038 ± 0.004 in controls) in the use of the downstream signals in the pseudodeficient individuals. It is unclear whether this represents variation in the level of GAPDH or ASA transcription in the four pseudodeficient individuals that were used in this study. Most of this variation can be attributed to a single pseudodeficient individual, (analysed in figure 4.4a, lane 4), where the 2.1 kb ASA mRNA contributed about 7% of the total amount of ASA mRNA. The protected RBNP bands which were quantified in this lane were extremely faint and there quantification may have been beyond the capability of the ImageQuant program.

Figure 4.8 Relative expression of the ASA mRNAs

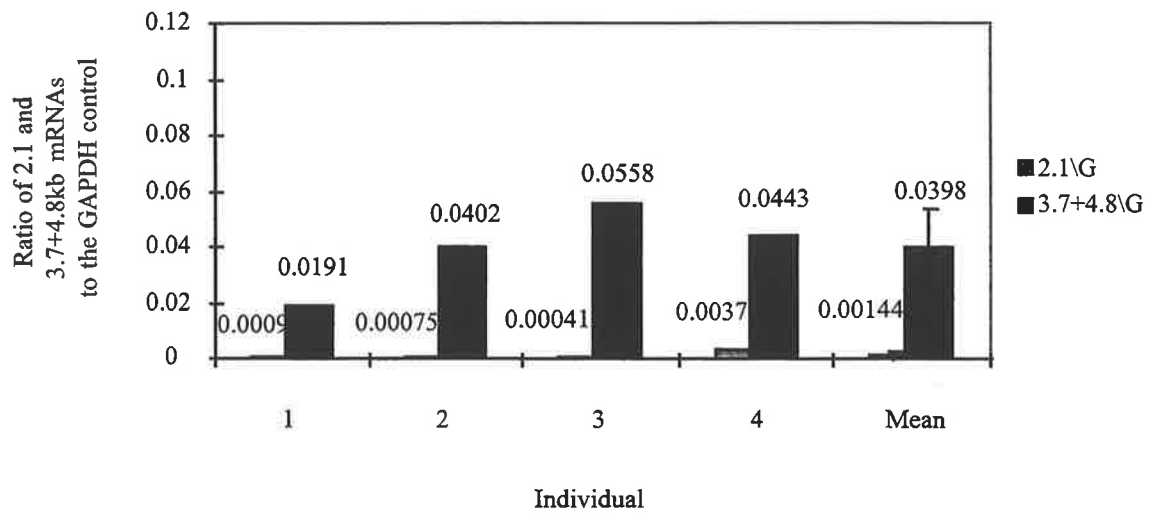
Transcription of the 2.1 kb and 3.7 + 4.8 kb ASA mRNAs relative to the GAPDH control. The ratio of the ASA transcripts to GAPDH has been calculated for each normal (a) and pseudodeficient individual (b) analysed in Figures 4.4a and 4.5. In Figure a, columns 1 to 3 represent the three normal individuals (1 to 3) analysed in Figure 4.5 and columns 4 and 5 represent the two normal individuals analysed in Figure 4.4a.

Figure b shows the results of the corresponding ratios for pseudodeficient individuals. Columns 1 and 2 represent the two pseudodeficient individuals (4 and 5) analysed in Figure 4.5 and columns 3 and 4 represent the two pseudodeficient individuals analysed in Figure 4.4a. The last column on each graph gives the mean and one standard deviation of the calculated ratios.

a



b



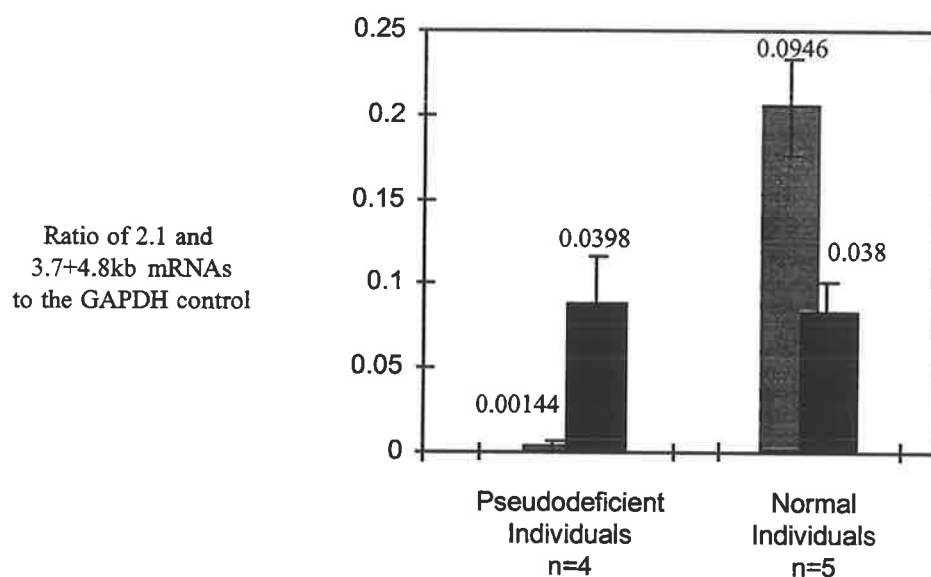


Figure 4.9 Comparisons of normal and pseudodeficient polyadenylation frequencies

The mean use of the 2.1 kb and 3.7 + 4.8 kb ASA mRNAs relative to the GAPDH control in pseudodeficient and normal individuals. Error bars represent one standard deviation from the mean in each case.

4.3.3 The Regulation of Steady State ASA mRNA Levels

The mechanisms which regulate the relative use of the three ASA polyadenylation signals are unknown. The work of Lagnado *et al.* (1994) on RNA instability elements however, provides some insight into one possible mode of regulation. Repeated AU-rich elements in the 3' untranslated region of several cytokine and oncogene mRNAs have been found to function as signals for the rapid degradation of mRNA. Such elements may conceivably govern the relative steady state frequencies of the differentially polyadenylated ASA mRNAs. As part of the HUGO project, chromosome 22 YAC clones containing the ASA gene sequence have been characterised (Collins *et al.*, 1995), although the sequences of these clones are not yet generally available. The identification of UA repeats or any other sequence elements which affected RNA stability in the region downstream of the 2.1 kb ASA polyadenylation site would suggest that the stability of the ASA mRNAs does play an important role in defining the overall *in vivo* levels of ASA mRNA available for translation.

In attempting to explain the steady state levels of the ASA mRNAs in terms of transcript stability, there are numerous assumptions which should be considered. In simplistic terms, this study has shown that for every 100 ASA transcripts synthesised, about 70 are polyadenylated by the 2.1 kb site and 30 by the 3.7 and 4.8 kb sites in normal individuals. In pseudodeficient individuals only 1 or 2 of the hypothetical 100 ASA mRNAs are polyadenylated at the 2.1 kb site, all other transcripts must therefore extend past this site and continue towards the downstream polyadenylation signals. Despite the increase in the number of mRNAs extending beyond the 2.1 kb signal, only about 30 of the 98 or 99 mRNAs are polyadenylated by the downstream signals, a figure almost identical to that in normal individuals. This raises the question of what happens to these extra transcripts and how can they be accounted for in a model to explain ASA mRNA levels in terms of transcript stability?

To suggest that instability elements downstream of the 2.1 kb ASA polyadenylation site regulate the steady state level of the 3.7 and 4.8 kb transcripts means that the downstream sites would have to be the major sites of transcription termination for the ASA gene. Gieselmann *et al.* (1989) provided some evidence to suggest that this may indeed be true, the longer ASA mRNA transcripts were estimated to be more abundant than the 2.1 kb transcripts, and were thought to account for 60% to 70% of total ASA mRNA (although no details were provided to explain how this estimation was made) even though the 2.1 kb mRNA was the most abundant polyadenylated ASA mRNA species. A model which would account for these observations in terms of inherent instability in the ASA mRNAs is presented in the following example.

If the 2.1 kb site is used in only 10% (70 transcripts) of ASA mRNA, and the remaining 90% (700 transcripts) are polyadenylated downstream, then to account for the steady state levels of polyadenylated ASA mRNA (ie 70% 2.1 kb and 30% 3.7 + 4.8 kb), the longer transcripts would have to be about 25 times more unstable than the 2.1 kb transcript. This would then account for the observed steady state ratio of the ASA mRNAs (7 to 3), since only about 30 of the original long transcripts would be available for translation. In the presence of the ASA-PD polyadenylation defect, 10% or 70 of the hypothetical transcripts would not be polyadenylated by the 2.1 kb signal, subsequently 770 transcripts would be available for polyadenylation downstream of this site. The inherent instability of the longer transcripts, as in normal individuals, would mean that only 31 of the original 770 ASA mRNAs would be available for translation. This would then account for the 30% of normal ASA mRNA levels observed in individuals homozygous for ASA-PD.

This model for the regulation of ASA transcript stability undoubtedly requires the accurate measurement of *in vivo* transcription as opposed to the analysis of steady state mRNA levels which are presented in this study. Newly transcribed RNAs can be detected

using nuclear runoff transcription assays, where cell nuclei are isolated and incubated with ^{32}P labelled UTP and cold NTPs to enable the specific labelling and subsequently the detection of nascent RNAs.

4.3 CONCLUSIONS

The results presented here indicate that both the N350S sequence variant and the polyadenylation defect, which together make up the ASA-PD allele, are important in biochemically defining ASA pseudodeficiency. The N350S ASA protein has reduced enzymatic activity in comparison to the normal clone and is inefficiently targeted to the lysosome. The combination of these two factors is expected to reduce the *in vivo* lysosomal ASA activity in pseudodeficient individuals by about 75%. The polyadenylation defect reduces the total amount of available ASA mRNA to about 33% of normal. The combined effect of these two sequence variants in a single allele is expected to reduce ASA activity to about 8% of normal, a value only marginally lower than that calculated by Gieselmann *et al.* (1989). Although our results basically concur with Gieselmann's original estimate of the effect of the ASA-PD allele in reducing ASA activity, the results presented do suggest an alternative explanation as to why this reduction occurs.

Publications which have supported the hypothesis that the N350S sequence variant is benign, do not necessarily contradict the findings of this work. An example of a publication supporting the original hypothesis put forward by Gieselmann *et al.* (1989) is that of Leistner *et al.* (1995). This recent paper documented the analysis of two families where individuals had different combinations of MLD mutations, N350S and the ASA-PD polyadenylation defect. One individual was identified as heterozygous for only the N350S. The reported ASA activity measured in this individual was within the normal range and the authors therefore concluded that N350S was benign.

In this case, the measurement of ASA from total cell lysates, negated any consideration of the location of the enzyme within the cell before assay, meaning that mistargeted enzyme as well as lysosomal ASA was measured. With this in mind, our measured reduction of the activity of the N350S expression construct (57% of normal) concurs with Leistner's observations since a single heterozygous copy of the N350S mutation would be expected to reduce ASA activity in this individual by only 21%. The presence of a single copy of N350S would not therefore alter an individual's classification within the normal range of ASA activities.

Our results, which demonstrate that N350S does play a role in reducing ASA activity, support the observations of both Shen *et al.* (1993) and Francis *et al.* (1993). Both authors identified MLD heterozygotes, who were also carriers of only the N350S sequence variant, with levels of ASA activity well below that expected for obligate ASA heterozygotes. In view of results presented here, these observations are not surprising since the single functional N350S allele would produce only about half of the expected ASA activity (28% of normal) measured in an individual who is heterozygous for an MLD mutation.

Chapter 5

Complex ASA Alleles and the Causation of MLD

5.1 INTRODUCTION

5.5.1 The ASA Activity Threshold

Under normal conditions, cells contain far more lysosomal enzyme than what is required to avert substrate accumulation. This is exemplified by the existence of the ASA pseudodeficiency allele for example, which causes a dramatic reduction in the activity of ASA (exceeding 90%) without causing the accumulation of substrate or the onset of disease. The question of what level of ASA activity separates normal development from the onset of MLD is important when trying to predict the clinical outcome which is associated with the inheritance of a particular genotype.

Conzelmann and Sandhoff (1983/84) put forward a simple model to correlate the residual activity of an enzyme and the presentation of a lysosomal storage disorder, by relating the rate of influx of a lysosomal substrate to its subsequent rate of degradation within the lysosome. The threshold level of lysosomal hydrolase activity which is required to avert the onset of the clinical symptoms of a disorder, was defined as the level of activity at which the rate of influx of a substrate into the lysosome equalled the ability of an enzyme to degrade that substrate.

Within this thesis the ‘threshold level of ASA activity’ refers to the amount of residual ASA activity that is just sufficient to avert the accumulation of cerebroside sulphate within nervous tissue and hence the onset of MLD.

5.1.2 Biochemical Phenotype and Clinical Presentation

The usefulness of the measurement of ASA activity, as a tool for predicting the severity of disease, or whether an individual will develop MLD, is limited by the inherent variability of the assay of the enzyme. When hypothesising a relationship between the a

measurement of residual ASA activity and the presentation of MLD, it is important to be aware of some of the factors which effect the accuracy and repeatability of the assay of the ASA.

5.1.2.a Tissue culturing factors

Carey and Pollard (1977) and Simpson and Kalow (1963), recognised the influence that tissue culturing factors could have on the *in vitro* activity of lysosomal enzymes. The authors observed variation in the activity of lysosomal hydrolase enzymes between subcultures of a single cell line.

Young *et al.* (1975) made a similar observation when studying variability in the activity of the lysosomal enzymes in skin fibroblasts and amniotic fluid cells. However, Young *et al.* (1975) recognised that although the activity of the lysosomal enzymes varied greatly between subcultures of a given cell line, the variability in the activity of all of the lysosomal enzymes measured was similar, ie the increase in the activity of one lysosomal enzyme was mirrored by a rise in the activity of others. This observation implied that the use of an internal control, where the expression of one enzyme was calculated as a proportion of another, could compensate for the apparent discontinuity in assay results detected between cultures.

5.1.2.b ASA activity and the severity of MLD

Carey and Pollard (1977) concluded from their study of the variation of the lysosomal hydrolases in culture, that it was often not possible to differentiate between the different clinical subtypes of a disorder on the basis of enzyme assays. The amount of residual ASA activity that was found to separated the clinical presentation of juvenile and adult MLD for

example, was smaller than the observed variability of the assay. This inability of the assay of ASA to differentiate between MLD subtypes has been recognised by many other authors (reviewed by Gieselmann *et al.*, 1994). ASA assays can however, usually differentiate between the undetectable level of ASA activity which characterises the late-infantile form of MLD and the low residual activity which characterises the later onset forms. (Leinekugel *et al.*, 1992).

The fact that the clinical subtypes of MLD are differentiated by small differences in ASA activity has considerable consequence for this study. This observation implies that at a pathogenic level of ASA activity, a further slight change in the activity of ASA could have a large effect on the clinical presentation of MLD. This in turn, may support the hypothesis that the multiple sequence variants identified in our patient population, have a role to play in the development of disease, even if these sequence variants only slightly effect ASA activity. The question arises for example, of whether a sequence variant which halved ASA activity, would be important in the presentation of an individual who was a compound heterozygote for the ASA-PD allele and a severe MLD mutation, if the sequence variant was inherited with the pseudodeficiency allele. These individuals have the lowest known non-pathogenic level of ASA activity, yet the amount by which this exceeds the threshold of level of ASA activity required to avert the clinical onset of MLD is unknown. Very small reductions in the activity of the ASA enzyme in such individuals could be critical to the development of disease.

5.1.3 Specific Aim

The establishment of a reliable and repeatable system for the *in vitro* assay of ASA and of specific ASA sequence variants is of primary importance if the relative effect of those specific alterations on the activity of the enzyme and the presentation of MLD are to be determined. This chapter presents the results of the establishment of such an assay system,

while addressing the inherent variability previously described in the *in vitro* assay of the lysosomal enzymes.

This use of this assay system has allowed the estimation of the threshold level of ASA activity required to cause disease and has also allowed the analysis of the effect that the various novel ASA sequence variants have on the activity of the ASA enzyme and on disease presentation. The role played by the complex combinations of ASA sequence variants identified in the patient population in the causation of disease has also been investigated.

5.2 SPECIFIC MATERIALS AND METHODS

5.2.1 Specific Materials

5.2.1.a Enzymes

T4 DNA polymerase	Boehringer Mannheim, Germany
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5.2.1.b Antibiotics

Geneticin [®] (G-418 sulphate)	Gibco BRL, Gaithersburg, MD, USA
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5.2.1.c Miscellaneous fine chemicals

Percoll [®]	Pharmacia Biotech, Uppsala, Sweden
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5.2.1.d Buffers and solutions

Percoll [®] stock solution	A solution of 1.0 M sucrose, 40 mM HEPES was diluted 1 in 4 with Percoll [®] giving a stock solution of 75% (v/v) Percoll [®] in 0.25 M sucrose, 10 mM HEPES pH 7.0
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5 x Polymerase mix	100 mM Tris HCl, 10 mM DTT, 50mM MgCl ₂ , 2.5 mM of dATP, dTTP, dGTP and dCTP, 5 mM ATP, pH 8.0
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5.2.2 Specific Methods

5.2.2.a Construction of the ASA expression clone

The pRSVN.07 expression vector was restricted with *Hind*III and *Not* I and the HT14/CP8 pBluescript[®] clone containing the ASA cDNA was restricted with *Nco* I and *Not* I. Oligonucleotides encoding the rat preproinsulin 5' untranslated sequence were synthesised with *Hind*III/*Nco* I sticky ends and hybridised together by mixing equal molar amounts of

each oligonucleotide, heating the mixture to 70°C and allowing the mixture to cool to room temperature. The three fragments were ligated in a single reaction. The ASA cDNA 5' untranslated sequence were present in equal molar amounts, while being in three molar excess of the vector DNA.

5.2.2.b Transfection of Chinese hamster ovary cells

ASA expression constructs were transfected into Chinese hamster ovary cells using DOTAP transfection reagent (Boehringer Mannheim, Germany) under conditions recommended by the supplier.

5.2.2.c Cloning of the ASA cDNA into M13mp19

The ASA cDNA, HT14/CP8, was directionally cloned into M13mp19 vector. After restriction of the vector and insert plasmids with *Kpn* I and *Sac* I, the vector and insert bands were gel purified and ligated with the insert band in 3 molar excess.

5.2.2.d Preparation of single stranded M13 DNA

The plating and propagation of bacteriophage M13mp19 was carried out as described by Sambrook *et al.* (1989). Large scale preparations of M13 single stranded DNA were prepared from 5 hr, 250 ml cultures of phage infected DH5 α F'. Cells were harvested at 4000 g for 15 min at 4°C and the supernatant was carefully removed and retained. PEG 8000 (10 g) and 7.5 g of NaCl were added to the supernatant and the solutes dissolved by stirring for 45 min at room temperature. The precipitate was recovered by centrifugation at 10000 g for 20 min at 4°C and resuspended in 10 ml of 10 mM Tris HCl pH 8.0 followed by extraction with an equal volume of buffered phenol. The extraction was repeated with phenol : chloroform

(1:1) and the aqueous phase transferred to a fresh tube. The DNA was precipitated by the addition of EtOH and resuspended in 500 μ l of H₂O.

5.2.2.e Oligonucleotide mediated mutagenesis

A 20 ng sample of synthetic oligonucleotide encoding a mutant ASA sequence was phosphorylated in a 20 μ l reaction containing 1 x polynucleotide kinase buffer and 5 U of T4 polynucleotide kinase for 60 min at 37°C. The reaction was halted by the addition of 3 μ l of 100 mM EDTA and the enzyme denatured at 70°C for 2 min. The kinased oligonucleotide was hybridised to the single stranded ASA cDNA template after the addition of 1.25 μ l of 20 x SSC and 1 μ g of single stranded M13 template to the kinase reaction. The mixture was placed in a 500 ml, 70°C water bath which was allowed to cool to room temperature. The reaction mix was then centrifuged briefly to collect any condensate and left to chill on ice before the addition of 20 μ l of 5 x polymerase mix, 2.5 U of T4 DNA polymerase and 2 U of T4 DNA ligase in a final reaction volume of 100 μ l. The mixture was incubated at 0°C for 5 min, room temperature for 5 min and finally at 37°C for 2 hrs before termination of the reaction by the addition of 5 μ l 0.5 M EDTA. A 10 - 15 μ l sample of the mutagenesis mix was transformed into competent DH5 α F' cells and plated as outlined in 2.3.13.d.

Resulting mutagenesis plaques were lifted onto nylon membranes and the duplicate lifts were screened for the presence of the mutated ASA sequences using allele-specific oligonucleotides for the normal and mutant ASA sequence, as previously described.

5.2.2.f Generation of mutant ASA expression constructs

Replicative form (RF) M13 DNA was prepared from plaques containing mutant sequences of interest. The mutated ASA/M13 clone and the normal ASA pRSVN.07 expression clone were subsequently restricted with *Cla* I and *Not* I to generate a DNA

fragment which contained all mutagenised ASA sequences. The mutant ASA/M13 sequence insert was ligated into the normal expression clone to produce the mutant expression vectors.

5.2.2.g Expression of mutant ASA constructs

pRSVN expression clones encoding the normal and mutant ASA cDNAs were transfected into Chinese hamster ovary (CHO) cells. Transfected cells were selected for G418 resistance and a mass culture of resistant clones was cultured for assay. Once a culture had reached confluency in a T75 tissue culture flask, the culture was subcultured at a split ratio of 1 in 3. The fresh cultures reached confluency in 2 days, after which they were left in a stage of quiescent growth for a further four days without changing the media, before the cells were assayed for ASA activity.

5.2.2.h Percoll[®] fractionation of cellular organelles

Cells from a confluent T75 flask of CHO cells containing expression constructs of interest were harvested and the cells transferred to a sterile 10 ml centrifuge tube. The cells were pelleted at 700 g for 3 min at 4°C. The cell pellet was resuspended and rinsed twice in 10 ml of PBS and pelleted by centrifugation.

The resultant CHO cell pellet was resuspended in 1.5 ml of 0.25 M sucrose and the suspension was drawn into a 10 ml syringe through a 23 G needle. All air was evacuated from the syringe and a paraffin wad was placed over the syringe tip. The plunger of the syringe was then withdrawn and released 6 times to release the cellular organelles into solution and the organelle mix was centrifuged at 100 g for 10 min at 4°C, the supernatant was removed and retained. The process was repeated and the supernatant were combined. This post nuclear supernatant was diluted with 17 ml of 0.25 M sucrose, 10 mM HEPES pH 7.0 and centrifuged for 15 min at 7500 rpm for 15 min at 4°C in a Beckman JA20 rotor. The

resulting granular fraction (pellet) was resuspended in 3 ml of 0.25 M sucrose, 10 mM HEPES.

Percoll[®] stock solution was diluted 1 in 4.16 with 0.25 M sucrose, 10 mM HEPES to give a final concentration of Percoll[®] in solution of 18% (v/v). The 3 ml granular fraction was layered over 17 ml of 18% Percoll[®], 0.25 M sucrose, 10 mM HEPES and the solution fractionated at 19500 rpm in a Beckman JA20 rotor for 60 min at 4°C. Fractions of 950 ul were collected from the density gradient for β -hexosaminidase and ASA assays.

5.3 RESULTS AND DISCUSSION

5.3.1 The *in vitro* Expression of ASA

5.3.1.a *Establishing an in vitro ASA assay system*

The ASA cDNA was cloned into the eukaryotic expression vector pRSVN.07 as described in 5.2.2a. Briefly, the ASA cDNA, HT14/CP8 was restricted from the plasmid vector pBluescript (Stratagene) with the restriction endonucleases Nco I and Not I (Boehringer Mannheim). This fragment contained the entire ASA coding and 3' untranslated sequences whilst excluding the 5' untranslated region. Oligonucleotide primers encoding the rat preproinsulin 5' untranslated sequences (Figure 5.1a) were ligated into the pRSVN.07 expression vector along with the ASA cDNA (Figure 5.1b) and transfected into MC1061 cells. Transformants were subsequently selected for ampicillin resistance.

Grunstein analysis (Grunstein and Hogness, 1975) was used to isolate transformants which contained both the ASA cDNA and 5' rat preproinsulin sequences as shown in Figure 5.2. Only two of 70 ampicillin resistant colonies (35 and 61) were found to contain both sequences. The plasmid DNA from both of these colonies was prepared and transfected into Chinese hamster ovary (CHO) cells using the DOTAP transfection method (Boehringer Mannheim) and selected for G418 resistance. Cultures grown from resistant clonal cells were assayed for the presence of the ASA gene product using the artificial substrate p-nitrocatechol-sulphate (Baum *et al.*, 1959).

CHO cells were chosen for the expression of the ASA constructs not only because of their rapid growth rate but also because these cells had no detectable background level of ASA expression. In contrast, CHO cells transfected with the normal ASA cDNA expression constructs, 35 and 61, contained easily measurable amounts of ASA activity.

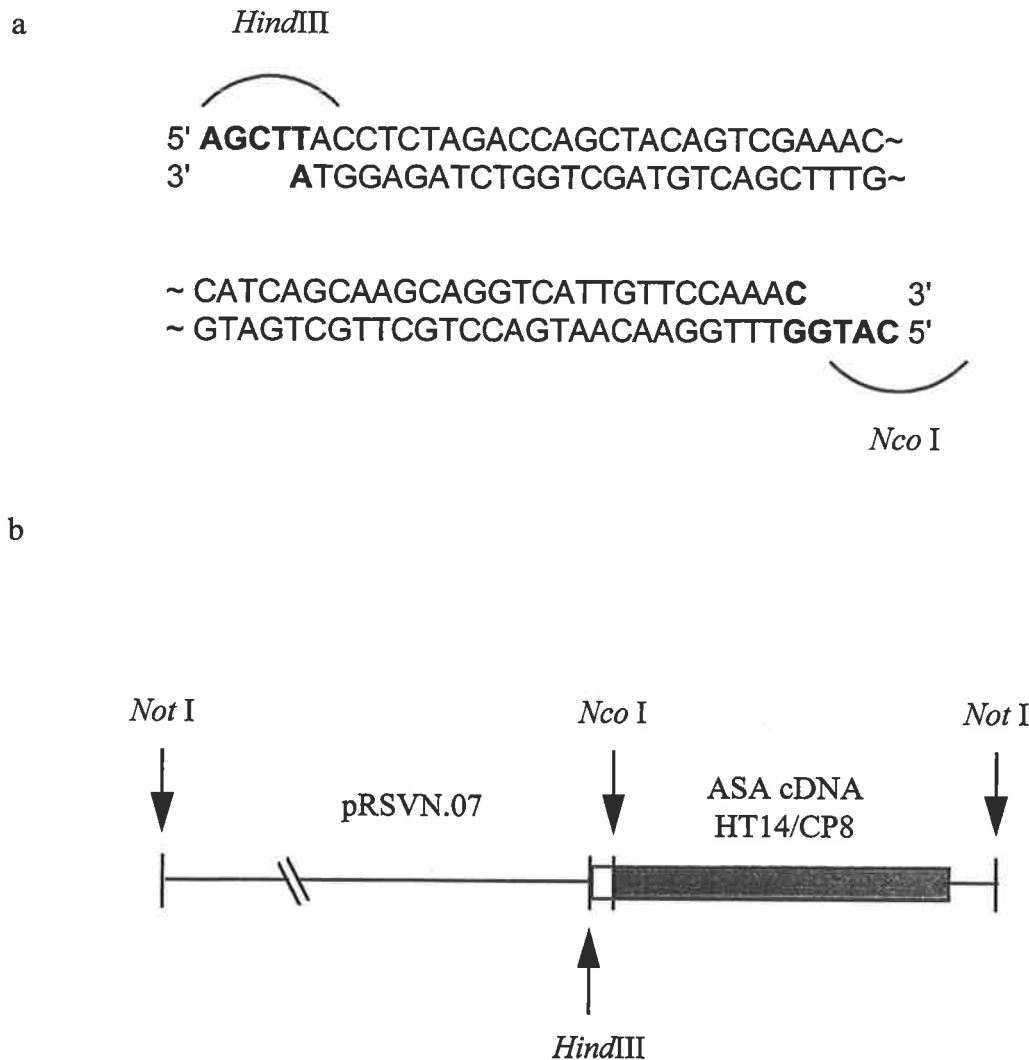


Figure 5.1 Construction of the ASA expression vector

The ASA cDNA HT14/CP8 (Stein *et al.*, 1989) was excised from the pBluescript vector with the restriction endonucleases *Nco* I and *Not* I. Oligonucleotides encoding the rat preproinsulin 5' untranslated sequences and containing a 5' *Hind*III site and a 3' *Nco* I site were synthesised and annealed (a). The ASA cDNA and 5' untranslated rat preproinsulin sequences were ligated, in a single reaction into the *Hind*III/*Not* I restricted pRSVN.07 expression vector (b). In Figure b, the shaded area represents the ASA cDNA and the open box, the 5' untranslated sequence.

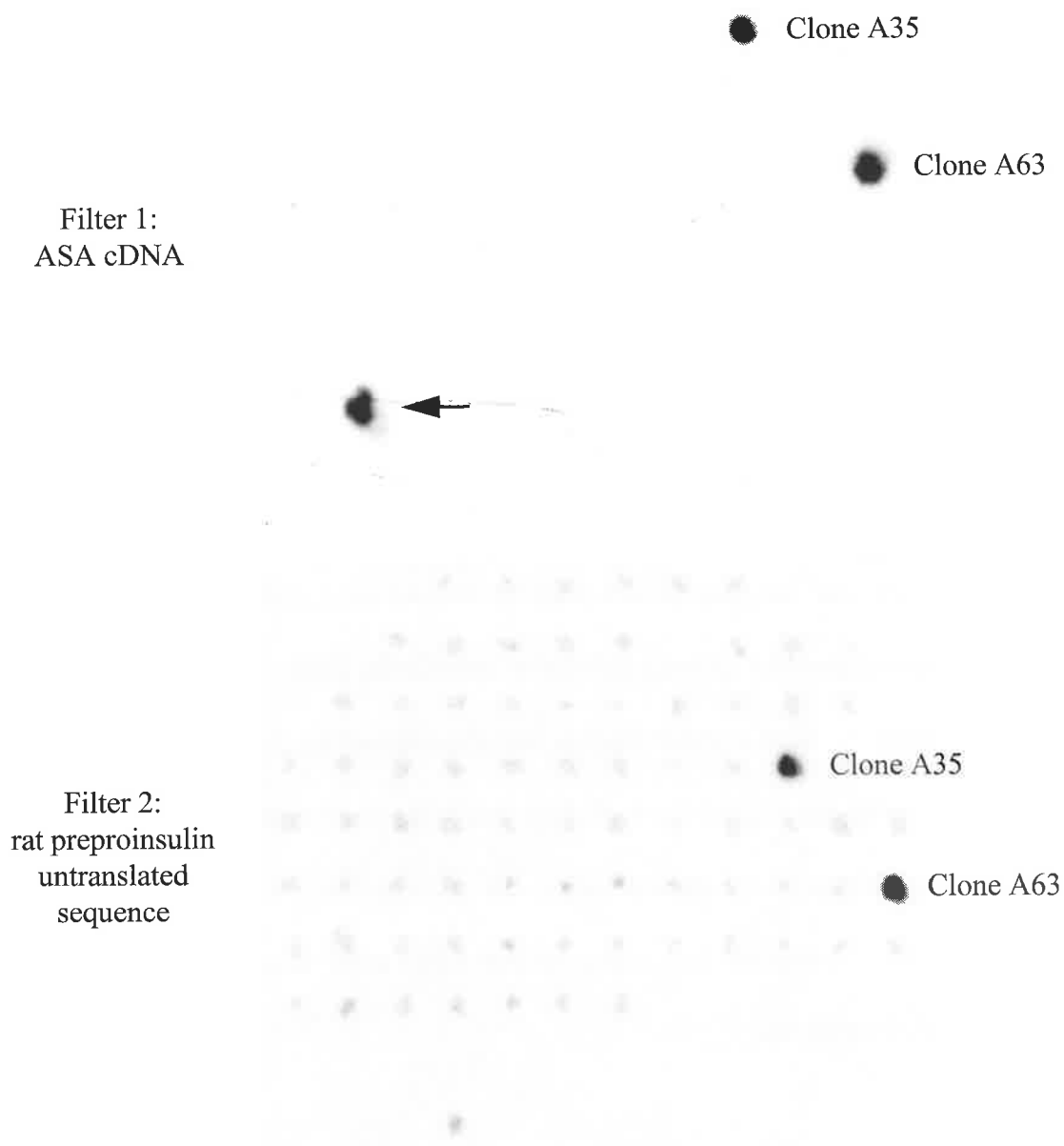


Figure 5.2 Analysis of the ASA expression vector

Filters 1 and 2 are duplicate Grunstein membranes containing DNA from 80 recombinant, ampicillin resistant cell colonies produced from the transformation of the ASA expression vector ligations products into DH5 α F' cells.

The duplicate membranes were hybridised with oligonucleotides detecting the ASA cDNA (a) and rat preproinsulin 5' untranslated sequence (b). A positive control for the ASA cDNA is arrowed.

The A35 and A61 plasmid constructs expressed ASA at a level of 880 and 609 nmol/min/mg of protein respectively, this is a level of expression approximately 10 to 50 times higher than that measured in normal skin fibroblasts (Figure 5.3).

The *in vitro* expression of the normal ASA gene construct required the use of the CHO cell cellular machinery to transcribe and translate the ASA expression construct products. The targeting of newly synthesised ASA to the lysosomal compartment is an essential step in the post-translational processing of the enzyme. In an attempt to gauge the ability of CHO cells to cope with the *in vitro* expression of the ASA cDNA construct, cellular organelles from a confluent flask of CHO cells expressing the normal (A35) ASA expression construct were fractionated on a Percoll[®]/sucrose density gradient (Brooks *et al.*, 1991). Each fraction was assayed for β -hexosaminidase (β -hex) activity to specifically detect lysosomal fractions (as described in chapter 2). Each cellular fraction was also assayed for the activity of ASA. The maximum level of enzyme activity for both ASA and β -hex was found in the 20th most dense gradient fraction (Figure 5.4), demonstrating the presence of cellular ASA within the lysosomes and thus indicating the ability of the CHO cells to process and target the overexpressed ASA correctly.

5.3.1.b *Mutagenesis of the normal expression construct*

An oligonucleotide based mutagenesis protocol was used to introduce specific alterations into the ASA coding sequence. Oligonucleotides encoding the mutant sequences of each novel allele were hybridised to an M13/ASA cDNA template and used to produce a double-stranded molecule from the single-stranded M13 template (as described in Chapter 2).

<u>Construct</u>	<u>ASA Activity</u> <u>nmol/min/mg</u>
A35	880.5
A61	609.1
CHO-K1	Not Detected (<0.01)

Skin Fibroblast Controls 5-50

Figure 5.3 Expression of the ASA cDNA construct

The ASA activity of clonal CHO cell expression lines containing the A35 and A61 plasmid expression constructs are presented. A35, A61, CHO-K1 control and skin fibroblast controls ASA activities were measured in flasks of confluent cells.

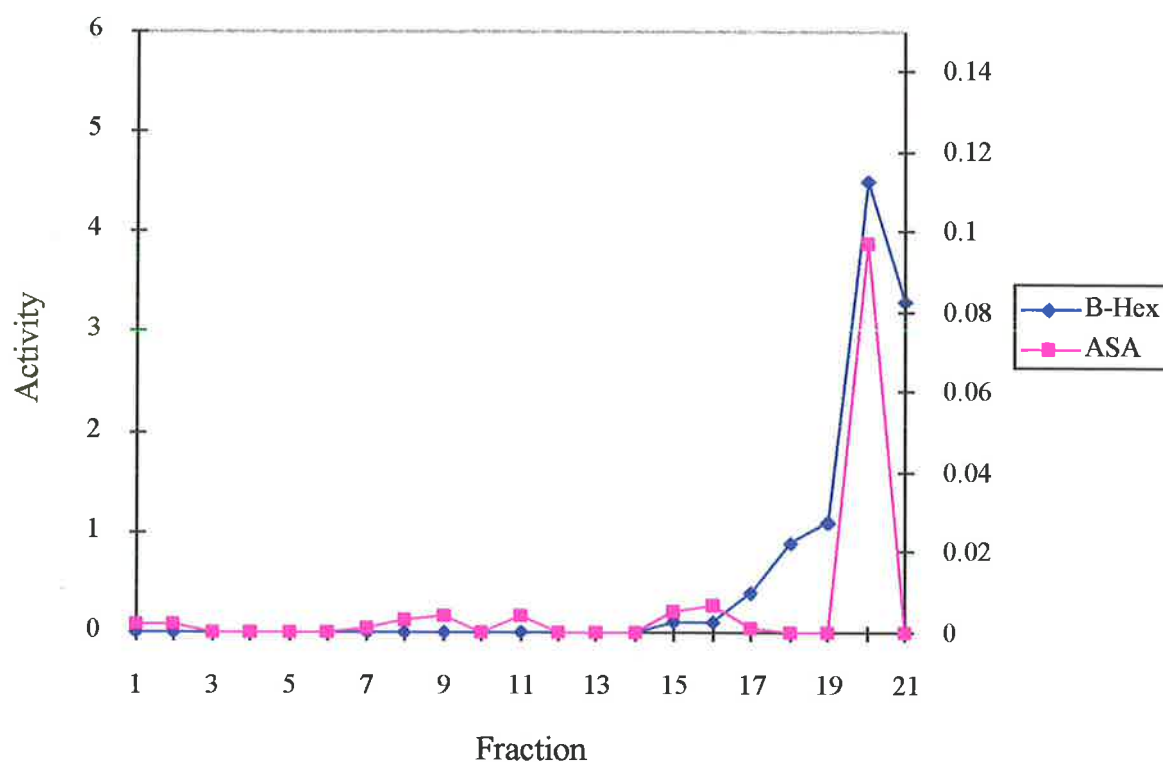


Figure 5.4 Targeting of ASA to the lysosome

Cellular organelle fractionations (1-21) from a Percoll[®]/sucrose density gradient, assayed for β -hexosaminidase and ASA activity.

Units on the left hand Y axis represents β -hexosaminidase activity in relative fluorescence units. The right hand Y axis represents ASA activity measured with the artificial substrate p-nitrocatechol-sulphate in units of absorbance at A_{515} .

Products of the mutagenesis reactions were transformed into DH5 α F' cells and plated onto L agar plates. Duplicate lifts were performed on the resulting plaques and the filters were probed with the oligonucleotide primers corresponding to the normal and mutagenised sequence, respectively (as described in chapter 2). Figure 5.5, shows the results of the hybridisation of the normal and mutant T391S oligonucleotides to duplicate filters containing S391 mutagenesis products. Theoretically, half of the plaques plated should be mutant and the other half normal, however in this case there are large numbers of parental plaques present. None of the mutagenesis products were produced at a frequency greater than 1% of the total number of plaques in the mutagenesis mix. Plaques identified as carrying the mutagenised copy of the gene were picked and confirmed as being mutant by ASO analysis of PCR products amplified from the plaque. Figure 5.6 shows the results of this analysis for S391 mutant plaques.

5.3.1.c Variability in the in vivo expression of ASA

Mass cultures of G418 resistant CHO cells were used for the expression of all ASA expression constructs. It was proposed that the use of mass cultures (as opposed to the use of clonal cells lines) would avoid problems likely to arise because of variability evident in the expression of ASA in clonal expression lines. The assay of the two normal ASA expression lines A35 and A61, for instance, gave different assay results even though they resulted from the expression of an identical plasmid construct. For the G418 resistance gene, and hence the ASA cDNA construct to be stably inherited, they must be incorporated into the genome of the cells in which they are expressed. The activity of the incorporated constructs must in part depend on where and how the plasmid is incorporated into the host CHO cell genome.

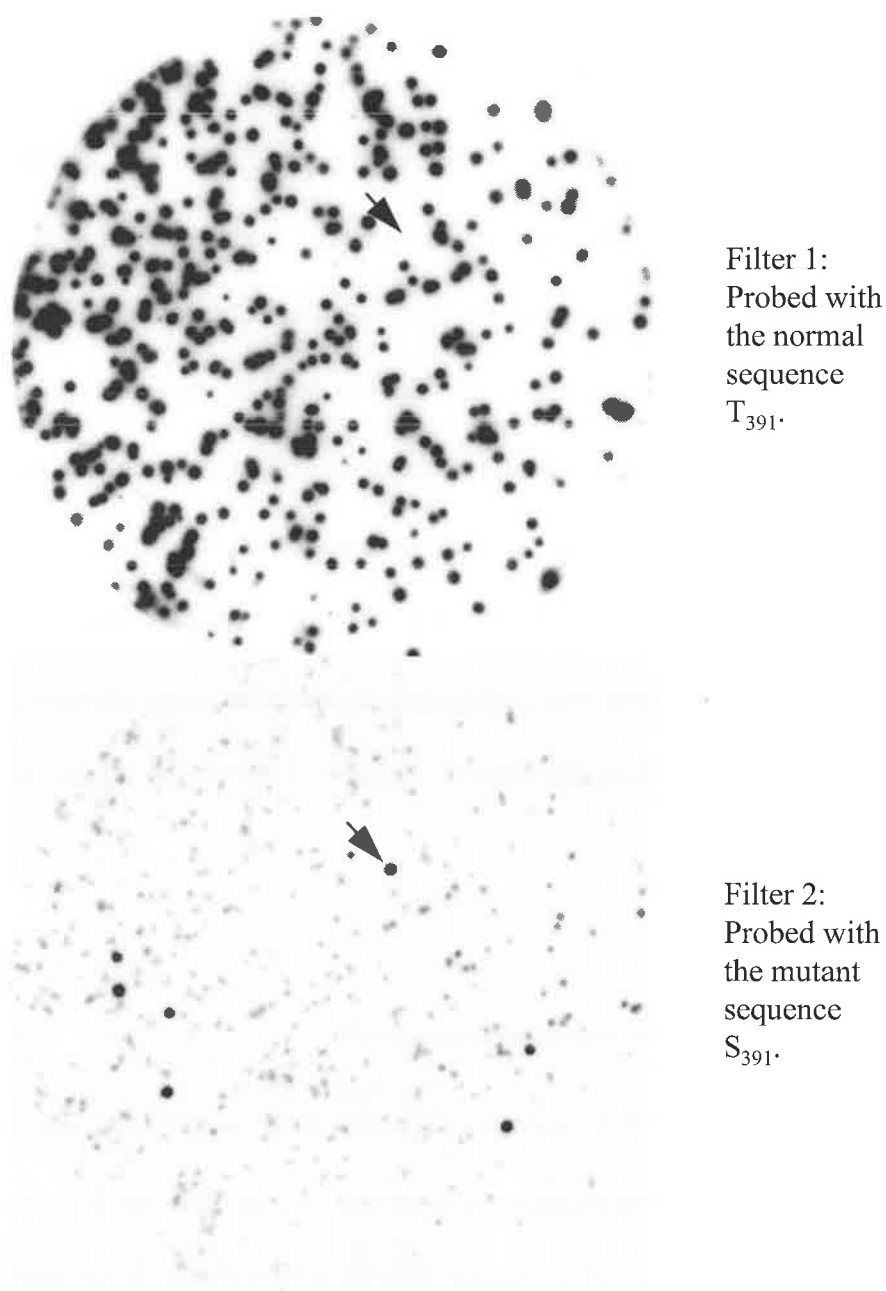


Figure 5.5 Analysis of mutagenesis products

Filters 1 and 2 are duplicate plaque lifts containing products from the T391S mutagenesis reaction, hybridised with the normal (T391) and mutant (S391) ASOs respectively. The arrow represents a plaque not bound by the normal T391 ASO and subsequently found to be homologous to the S391 ASO.

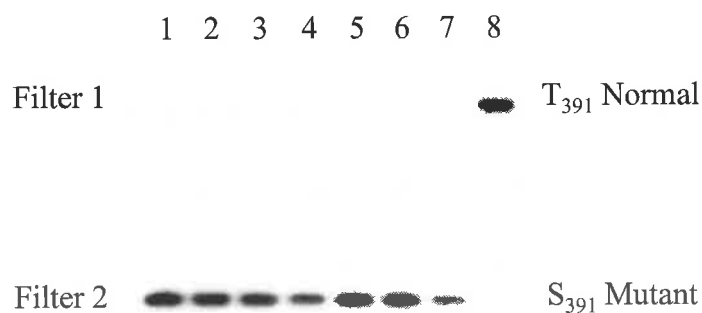


Figure 5.6 ASO analysis of mutagenesis plaques

Filters 1 and 2 are duplicates containing exon 7/8 ASA cDNA PCR products amplified from S391 mutagenesis plaques identified in Figure 5.5. Lanes 1 to 7 contain PCR products amplified from the mutant plaques, lane 8 contains a non-mutant control. Filters 1 and 2 were hybridised with the normal (T391) and mutant (S391) ASOs respectively.

An ASA expression construct containing the P155R sequence variant was used in conjunction with the normal construct, to develop the methodology for accurately measuring the relative activity of the mutant expression constructs. Figure 5.7 shows the results of the ASO analysis of the P155R mutagenesis and expression clones, the figure also highlights one of the difficulties in the production of mutant ASA clones. The R155 mutagenesis clone was isolated in a cluster of plaques and it was therefore difficult to pick the mutant without contaminating it with adjacent normal plaques. This is confirmed by the ASO analysis of the mutagenesis products which shows that the mutant plaque suspension also contained normal sequences (Figure 5.7a). Expression constructs produced from this mixture of normal and mutant P155R mutagenesis plaques were checked for the presence of the normal and mutant sequences by ASO analysis, to ensure that a single mutant clone was used in the expression analysis (Figure 5.7b).

The normal and P155R expression constructs were transfected into CHO cells as described in the Chapter 2. Mass cultures of G418 resistant colonies were grown to confluence only if more than 200 such colonies were produced from the transfection. Tissue culture cell populations pass through several stages of growth between subcultures. An initial stage of recovery where growth over time is non-linear is followed by an exponential growth phase before competition for space and resources restricts growth which ultimately plateaus and reaches a stage of quiescence (Paul, 1972).

ASA activity is calculated relative to total cellular protein. Since the composition of the protein content within actively dividing cells and those which are quiescent may vary, it is important, when comparing the activity of two cell lines, to ensure that each is in a similar growth phase. Normal and P155R expression lines were consequently assayed when the cells were in an exponential phase of growth (24 hrs prior to confluence) to ensure that their expression would be comparable. The expression of the mutant cell line was calculated as a

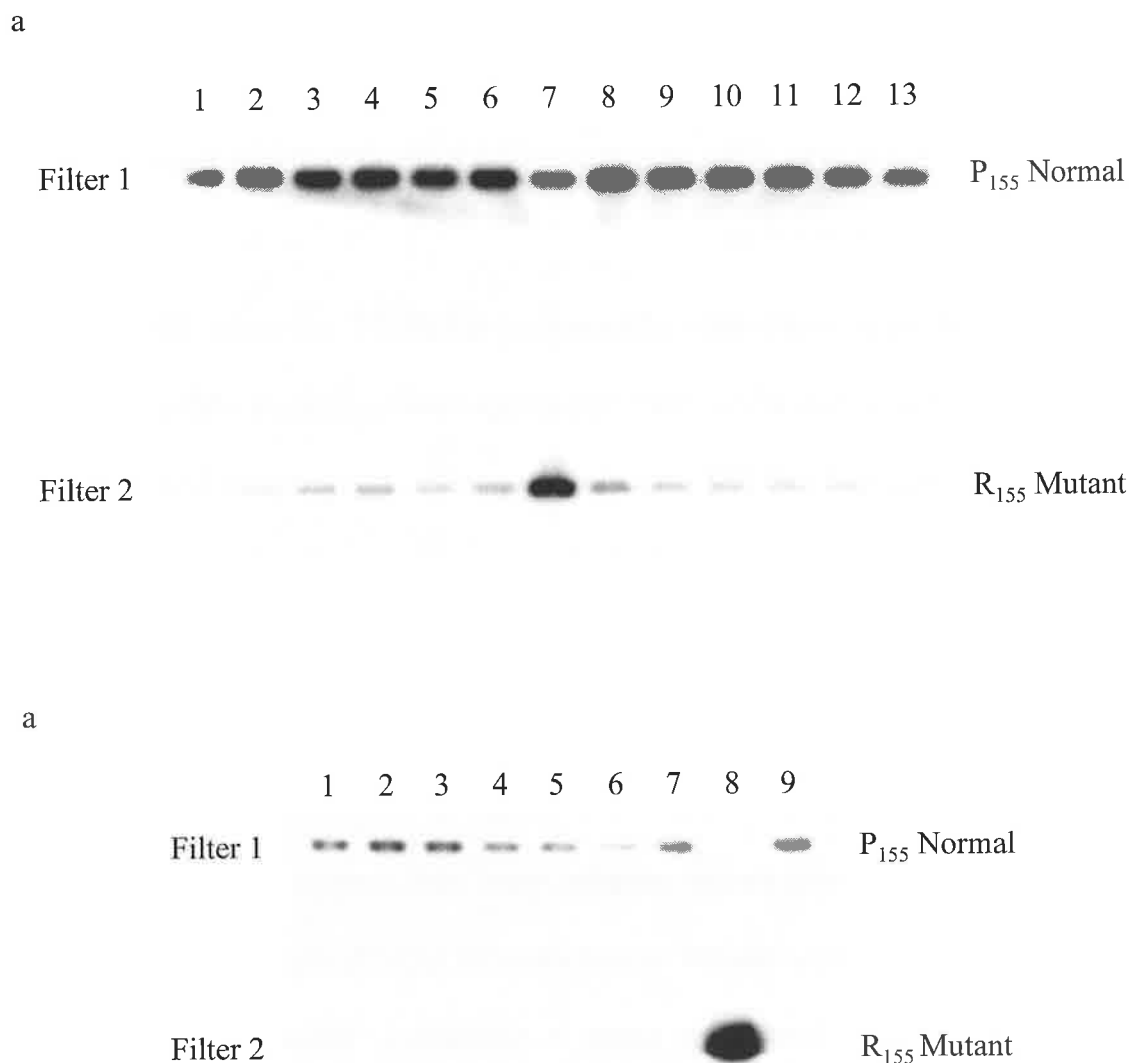


Figure 5.7 ASO analysis of P155R mutagenesis and expression clones

a) Filters 1 and 2 are duplicates containing exon 3/4 amplified cDNA PCR products from plaques picked from a P155R mutagenesis reaction. Filters 1 and 2 were hybridised with the normal and mutant P155R ASOs respectively. All plaques were picked (lanes 1 to 13) from a compact group containing a mutant P155R clone.

b) Analysis of exon 3/4 PCR products, amplified from pRSVN expression clones with the normal (filter 1) and mutant (filter 2) P155R ASOs. The lane 8 amplified clone was produced from the P155R mutagenesis product identified in lane 7 of (Figure 5.7a). Lanes 1 to 7 and 9 are controls.

proportion of that expressed by the normal ASA construct. It was thought that variability in the expression of the P155R construct due to culturing factors would be reduced by normalisation to the activity of the normal control cell line if both cultures were subcultured, grown and assayed synchronously.

Although the level of activity of the normal and P155R expression constructs did vary, a rise in the level of activity of one clone was not necessarily mirrored with a rise in the other. The relative activity of the P155R construct over twelve measurements (Appendix 6) varied widely around a mean of 8.7% ($\pm 4.5\%$) of normal (Figure 5.8). It is unfortunate that the observed degree of variability in the level of activity quite probably spanned both pathogenic and non-pathogenic levels of expression. It is therefore difficult to use this assay as a measure of the specific effect that the novel ASA alleles will have on the activity of the enzyme.

Eight of the above twelve samples were also assayed for ASA activity using sulphatide, the radioactively labelled natural substrate of ASA (Poulos and Beckman, 1981). The raw assay data from these assays appear in Appendix 7. The average level of activity of the P155R construct using this system of assay was $5.5\% \pm 3.5\%$ of normal (Figure 5.7b). Again, unfortunately the calculation of the construct's relative level of expression was crude and the variability in the measurements probably spanned both pathogenic and non-pathogenic levels of expression. These results cast doubt on the usefulness of the assay as a tool for the quantification of the effect of an allele on ASA activity. However, the suggestion emerged during these experiments that the variability in the relative activity of the P155R construct may be due to factors other than inherent variability of the assays since both ASA and sulphatidase activity varied widely. Subsequent assays were therefore performed on confluent cell cultures whose growth had reached a stage of quiescence. Expression lines were subcultured and grown for 3 days post confluency and assayed with the p-nitrocatechol-

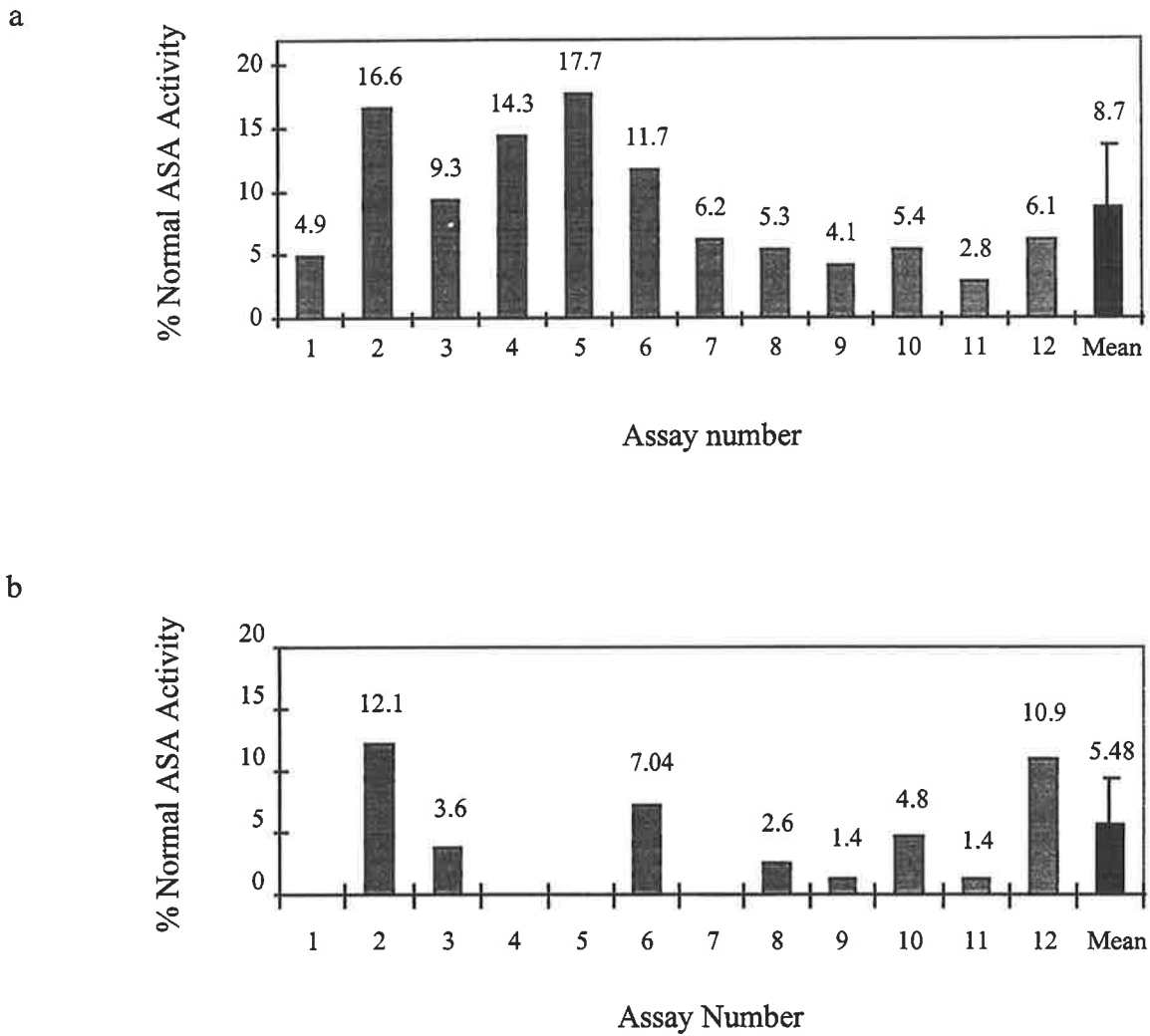


Figure 5.8 Normal and P155R expression in exponentially growing cells

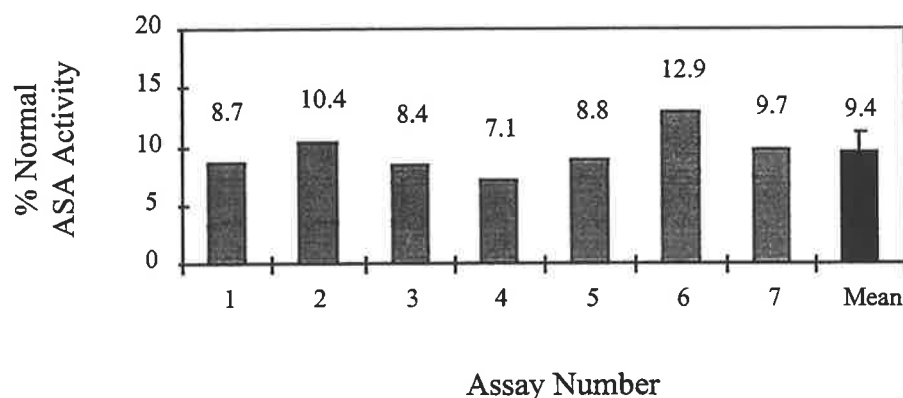
Figures 5.8 a and b show the relative activity of the P155R expression construct over 12 subcultures with the artificial and natural substrates of ASA respectively. The last column on each graph represents the mean of each sample with the error bars representing one standard deviation each side of the mean. Blank columns in Figure 5.8b represent samples which were not assayed. For each assay the measured level of ASA activity is expressed as a percentage of that measured in the normal expression construct.

sulphate substrate. The media on the cell lines was not changed during this time, which ensured that the cells had to compete for the available nutrients.

The level of ASA activity measured in the normal and P155R expression lines again varied greatly between different subcultures (Appendix 5.3) but in contrast to the previous assays, tended to vary synchronously, an increase or decrease in the measured level of activity of one construct was mirrored by a similar change in activity of the other. The variability of expression of the P155R construct relative to the normal was consequently markedly reduced compared to that of the two sets of assays performed on cells in the linear phase of growth (Figure 5.9a), although the mean level of the expression of $9.4\% \pm 1.8\%$ of normal, was close to that previously measured with the artificial p-nitrocatechol-sulphate substrate (8.7% Figure 5.9b). All subsequent expression constructs were therefore assayed using the above method.

It seems reasonable to conclude from these results that although the level of ASA activity within the CHO expression lines varied with the rate at which cell cultures grew (i.e. with their rate of exponential growth), the rate of expression of the mutant and normal constructs in the quiescent phase of growth where cells were not actively dividing seemed to be relatively stable. The expression construct was designed to express ASA by the use of a strong constitutive promoter, the rate of synthesis over time should not vary greatly because of this construction. The variability in the expression of the enzyme, as calculated as a proportion of total cellular protein, is most probably due to alterations in the specific population of proteins within the cell at different stages of growth. Additionally, even if two cell populations such as the normal and P155R expression lines are in a linear phase of growth, their actual rate of that growth need not necessarily be equal. This may have been a further cause of variability in the relative level of expression of the two cell lines when ASA activity was assayed in exponentially growing cells.

a



b

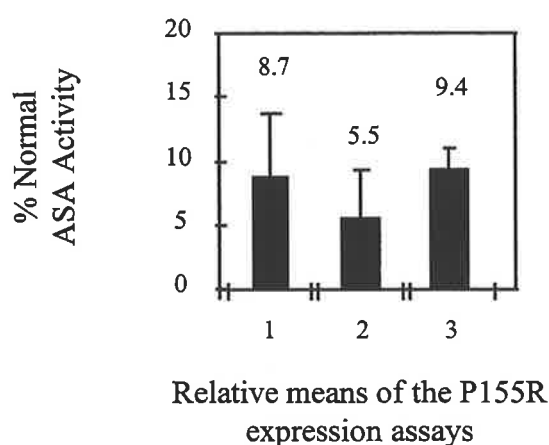


Figure 5.9 The relative expression of the P155R construct in quiescent CHO cells

a) Relative activity of the P155R expression construct over seven measurements of activity in quiescent cells. The final column on the graph gives the mean level of activity with the error bars representing one standard deviation from the mean. P155R activity is expressed as a percentage of that measured concurrently in the normal ASA expression cell line.

b) Comparison of the mean and variation in relative P155R expression under three different assay conditions. Columns 1 and 2 represent the expression of the construct measured in cells undergoing exponential growth with the artificial p-nitrocatechol-sulphate and natural sulphatidase substrates respectively. Column 3 shows the level of expression measured with the artificial substrate p-nitrocatechol-sulphate in quiescent cells.

In contrast, although different cell-lines and different subcultures of a cell-line reach their quiescent stage of growth at different rates, the rate of protein synthesis required to maintain cells at this quiescent stage seems to be relatively constant. The rate of synthesis of ASA relative to this background level of protein synthesis is subsequently less variable.

5.3.2 The ASA Activity Threshold

The level of ASA activity which separates normal development and the onset of MLD must, by deduction, lie between the lowest known level of ASA activity found in normal individuals and the highest level of activity identified in a patient affected by MLD, such patients will be among the mildest patients within the clinical spectrum of MLD.

Work presented in Chapter 4 established that individuals who are homozygous for the ASA pseudodeficiency (ASA-PD) allele have a level of activity which is about 8% of that expressed by a non-pseudodeficient control. Penzein *et al.* (1993), when investigating a possible link between the ASA-PD allele and the development of MLD, demonstrated that individuals who were compound heterozygotes for the ASA-PD allele and a severe (or null) MLD mutation such as 459+1G>A were not affected by the disorder. Half of the level of ASA activity measured in an ASA-PD homozygote (4.0%) must therefore be sufficient to prevent the *in vivo* accumulation of sulphatide and the onset of clinical symptoms. This level of activity represents the lowest known non-pathogenic level of ASA expression and hence represents the upper limit of the ASA activity threshold.

The estimation of the highest level of ASA activity found among MLD patients required the expression of a known mild or adult mutation, yet the question of just which mutation to express was one of some debate.

Analysis of the Australian MLD population for known pathogenic mutations, ascertained that the combination of the I179S mutation and the severe late-infantile allele, 459+1G>A resulted in the presentation of the adult form of the disease. The fact that I179S could compensate for the presence of the severe allele meant that it must produce a significant, although still pathogenic level of ASA activity. This allele was therefore a candidate for expression to estimate the lower limit of the ASA activity threshold.

The fact that no homozygous I179S individuals have been identified however, casts doubt on the ability of the allele to cause MLD in isolation. This allele may indeed simply be a pseudodeficiency allele that is only involved with the presentation of disease when combined with a severe MLD mutation. This hypothesis is supported by the findings of Fluharty *et al.* (1991) who after identifying I179S, transiently expressed the mutation and found that it expressed approximately 5% of normal ASA activity. Although this result was from a poorly controlled expression system, the reported level of expression does exceed our estimation of the upper limit of the ASA activity threshold and it therefore seems possible that I179S is non-pathogenic in isolation. The two adult MLD patients identified in this study as compound heterozygotes for the I179S and infantile 469+1G>A mutations would be expected to have about 2.5% of normal ASA activity, a level of expression which is presumably pathogenic.

The known adult MLD mutation P426L was chosen as the allele to be expressed to approximate the lower limit of the ASA activity threshold. It is one of the most common pathogenic MLD mutations. Affected P426L homozygotes have been identified and the ability of the allele to cause the adult form of the disease has therefore been confirmed.

The analysis of the products of the P426L mutagenesis reaction demonstrated, once again, one of the working difficulties of such a procedure. The picking of a single mutant colony from a densely plated population of plaques made it difficult to pick the mutant plaque

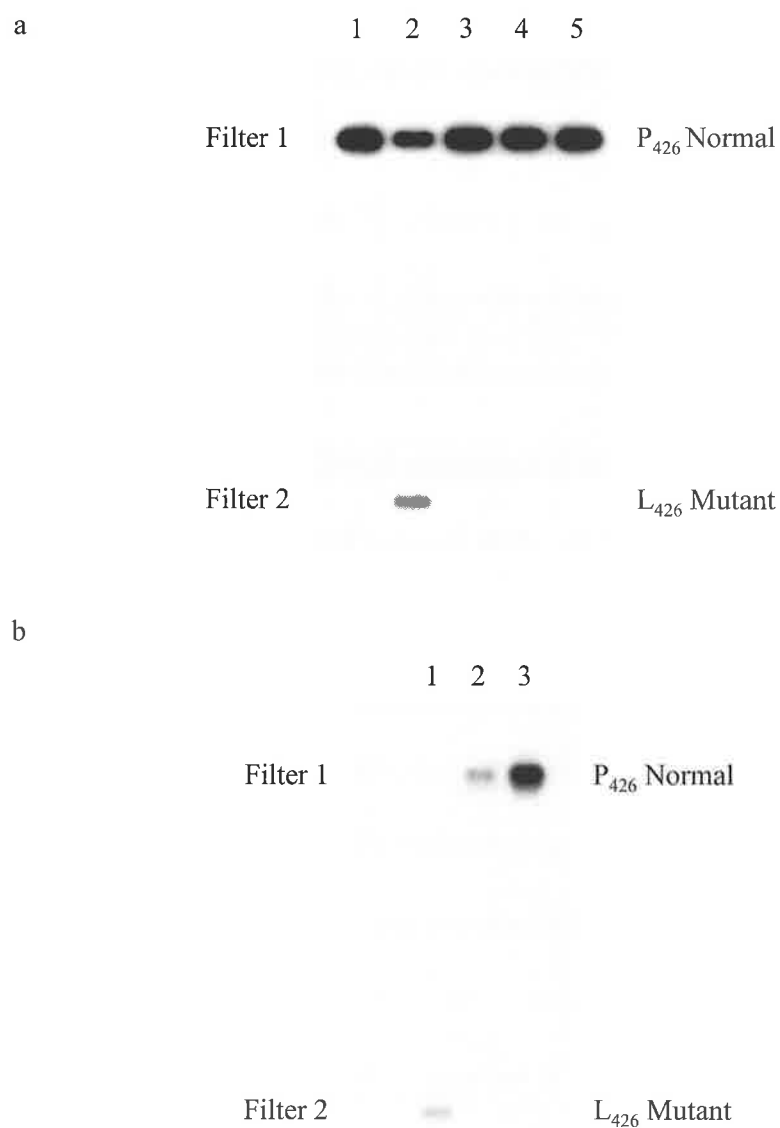


Figure 5.10 Analysis of the P426L mutagenesis clones

a) Filters 1 and 2 are duplicates containing exon 7/8 cDNA PCR products amplified from five adjacent mutagenesis plaques hybridised with the normal and mutant P426L ASOs respectively.

b) Analysis of duplicate filters (1 and 2) containing exon 7/8 cDNA PCR products amplified from P426L expression clones, hybridised with the normal and mutant P426L ASOs respectively.

without contamination from surrounding normal plaques (Figure 5.10a). Only a proportion of the P426L mutagenesis products ligated into the expression vector therefore contained the mutagenised sequence (Figure 5.10b).

Figure 5.11 presents the results of the P426L expression analysis and demonstrates that this mutation reduces the level of activity of the ASA enzyme to $2.4\% \pm 1.0\%$ of normal. This result places the threshold level of ASA activity required to cause the onset of disease between the 2.4% of activity expressed by the P426L allele and 4.0% of normal, the activity estimated to occur in compound heterozygotes for the ASA-PD and a severe MLD mutation. The I179S sequence variant could now almost certainly be classed as a pseudodeficiency allele since the likelihood of this sequence variant causing disease in isolation seems remote given its measured residual activity of 5% of normal. The 2.5% of normal activity estimated to be found in an I179S/469+1G>A compound heterozygote however, does fall within the pathogenic range defined in this study and would account for the presentation of these patients with a mild adult form of MLD.

5.3.3 Analysis of MLD Patient Genotypes

5.3.3.a Pseudodeficiency alleles and the presentation of MLD

Characterisation of the ASA-PD allele, (see Chapter 4) demonstrated that both the N350S variant and the polyadenylation defect which constitute the allele, play a role in biochemically defining the reduction in ASA activity observed in ASA-PD homozygotes.

The identification of the homozygous P155R sequence variant in patient MM who was also homozygous for the ASA-PD allele, aroused suspicion about the role of the ASA-PD allele in disease development. Expression of P155R revealed that it expressed $9.4\% \pm 1.8\%$ of normal activity, a level which exceeds the upper limit of the threshold of ASA activity

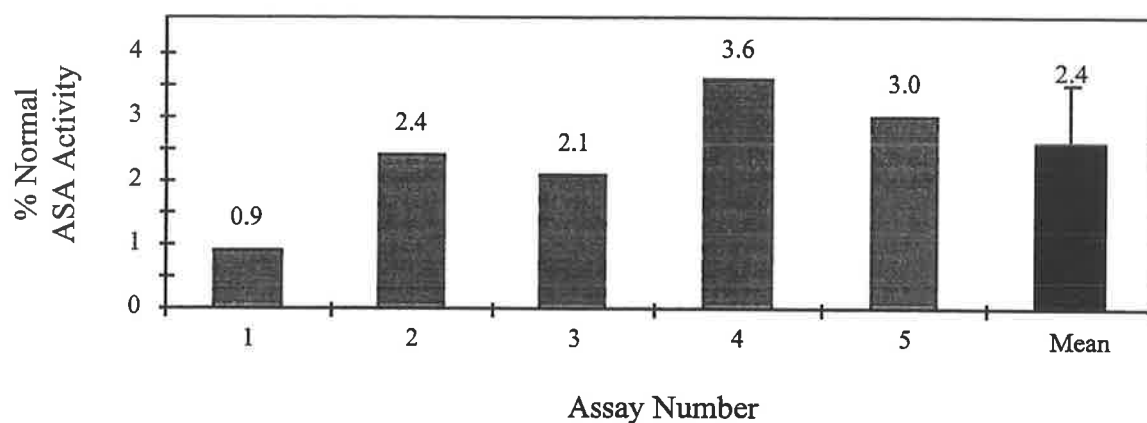


Figure 5.11 Establishing of the lower limit of the ASA activity threshold

Expression of the P426L construct measured as a percentage of normal in 5 synchronous quiescent cultures. The final column on the graph represents the mean level of activity measured from the construct, the error bar depicts one standard deviation from the mean.

required to cause disease. This finding implies that P155R, like the ASA-PD allele itself, would be non-pathogenic if found in isolation and therefore may be considered as 'benign'. An ASA allele containing the P155R sequence variant could therefore be classified as second ASA pseudodeficiency allele, since by definition, it lowers ASA activity to a level which would be difficult to differentiate from that of MLD patients.

Patient MM, to the best of our knowledge, is the first individual to be identified with an inborn error of metabolism, caused by the combined effect of two pseudodeficiency sequence variants on a single allele. ASA-PD and P155R, each reduce ASA activity to a similar level, indeed P155R expresses about 1.5% more activity than the ASA-PD allele. The combined effect of the presence of these two homozygous sequence variants on the same allele, would be to produce a residual ASA activity of less than 1% of normal, which would account for the presentation of this patient.

It is important to recognise that the combined effect of P155R and N350S amino acid substitutions on the same protein product need not necessarily be additive. A construct containing both of these alterations was expressed to allow the measurement of the combined effect of the two sequence variants within a single allele (Figure 5.12). As concluded from chapter 4, the N350S variant reduces the activity of the normal ASA expression clone by approximately half. Similarly, the N350S variant was found to reduce the activity expressed by the P155R construct by approximately half, from 9.4% to 4.3% of normal (Figure 5.13). Although there was some variability in the level of expression of the P155R/N350S clone ($4.3\% \pm 3.5\%$), perhaps due to the small number of repeat experiments, it appears that, in this case, the combined effect of the two variants on ASA activity is approximately additive.

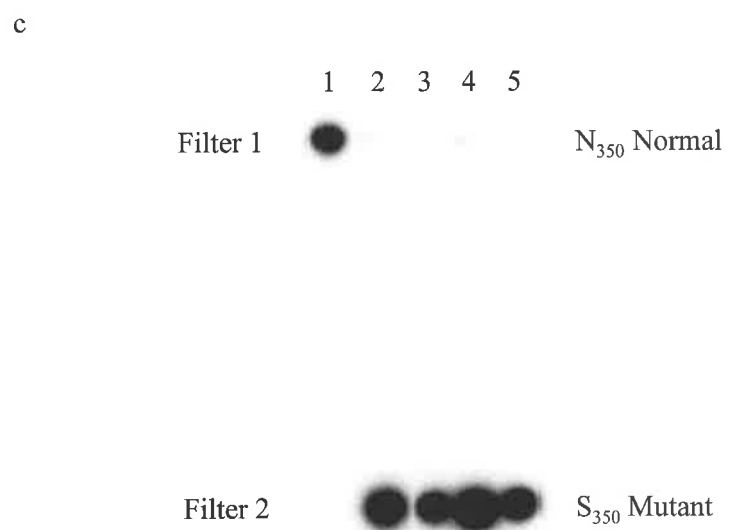
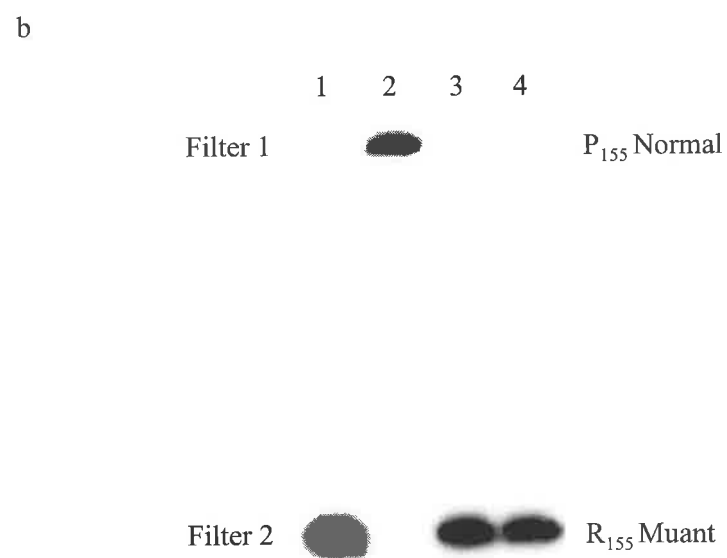
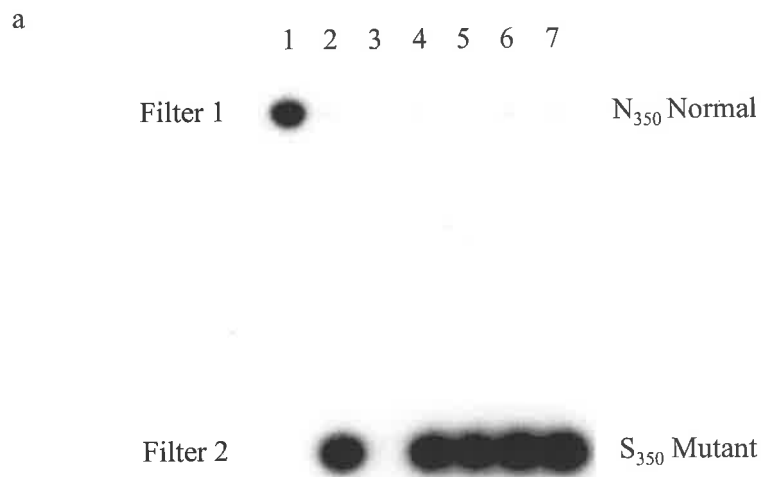
Due to the low level of residual activity expressed by the P155R/N350S mutant clone, it was not possible to investigate the targeting of the protein produced by the construct to the lysosome as the level of ASA activity in the Percoll[®]/sucrose gradient fractions, was too low

Figure 5.12 Construction of the P155R/N350S mutagenesis and expression constructs

a) Filters 1 and 2 are duplicates containing exon 5/6 cDNA amplified PCR products which have been hybridised with the normal and mutant N350S ASOs. Lanes 1 and 2 contain amplified products from the normal ASA/M13 and N350S ASA/M13 clones respectively, lane 3 is blank, lanes 4 and 5 contain P155R/N350S and lanes 6 and 7 contain D169N/N350S mutagenesis clones.

b) ASO analysis of P155R/N350S pRSVN expression clones. Duplicate filters 1 and 2 contain exon 3/4 amplified PCR products. Lanes 1 and 2 are the P155R and normal expression constructs respectively. Lanes 3 and 4 are P155R/N350S double mutant expression constructs.

c) Duplicate filters 1 and 2 containing exon 5/6 cDNA amplified products from the normal (lane 1), P155R/N350S double mutants (lanes 2 and 3) and D169N/N350S double mutants (lanes 4 and 5) hybridised with the normal and mutant N350S ASOs respectively.



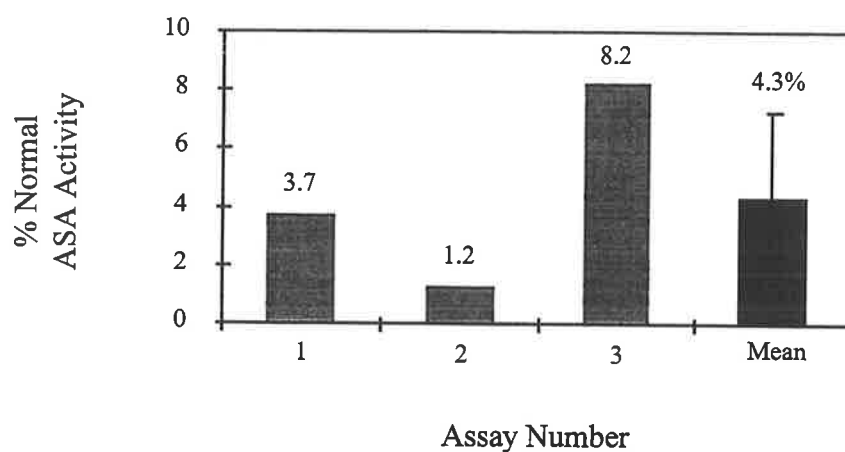


Figure 5.13 Expression of the P155R/N350S double mutant

Columns 1 to 3 represent the relative activity of the P155R/N350S expression construct in three duplicate experiments in quiescent normal and P155R cell lines. Column four of the graph gives the mean level of expression with the error bars representing one standard deviation from the mean.

to detect using the p-nitrocatechol-sulphate substrate. It is expected that the presence of P155R in the N350S expression construct would have no effect on the already aberrant targeting of the S350 enzyme to the lysosome since the P155 residue is not involved in lysosomal targeting. The possibility that the P155R mutation causes a subtle structural change that may affect the targeting of the N350S construct cannot be totally discounted but such an alteration might also be expected to affect the construct's affinity for its substrate and this is not suggested by the additive nature of the expression results.

5.2.3.b Genetic background and the presentation of MLD

The demonstration of four homozygous alterations within the ASA coding sequence of patient WT was puzzling, but presented a further opportunity to study the effect that a combination of sequence variants could have on the presentation of disease. WT was shown to be homozygous for the N350S and polyadenylation defects which constitute the ASA-PD allele, as well as the novel sequence variants, D169N and Δ F374.

The D169N and Δ F374 sequence variants (Figure 5.14) were expressed separately and, subsequently in combination, to identify any interaction between the two alterations. D169N reduced the activity of the ASA enzyme to $53\% \pm 19\%$ of normal and Δ F374 reduced activity to an almost undetectable level ($0.26\% \pm 0.16\%$ of normal) respectively (Figure 5.15). This analysis clearly implicates the deletion of amino acid number F374 as the primary pathogenic mutation in this patient, with the D169N sequence variant having little consequence for the presentation of the patient. Although inconsequential to the presentation of WT, the introduction of an asparagine residue to the ASA polypeptide via D169N could conceivably have an effect on the targeting of the enzyme to the lysosome. Figure 5.16 shows the results of the fractionation and assaying of Percoll[®]/sucrose gradient fractions for ASA and β -hex activity.

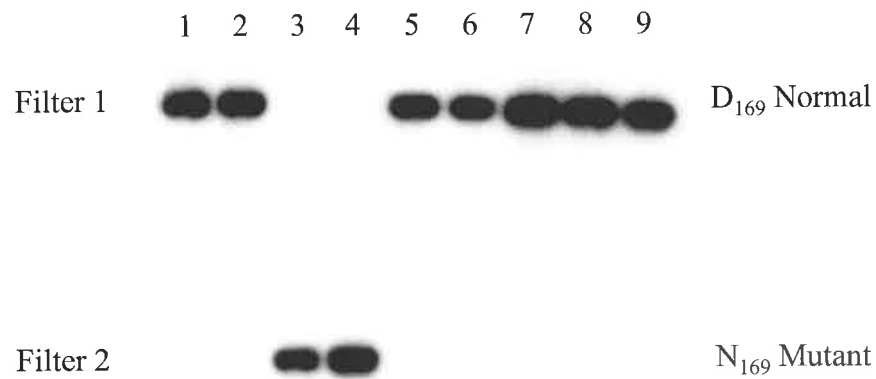
The presence of the N169 residue clearly has a detrimental effect on the targeting of ASA to the lysosome as approximately 35% of the ASA within the gradient is present in the non-lysosomal fractions, indicating that the enzyme is present in organelles other than the lysosome. The most likely organelle in which the enzyme might appear would be the endosomes, since mistargeted lysosomal hydrolases are usually channelled along the excretory pathway and expelled from the cell (von Figura and Hasilik, 1986). This fraction of ASA is not available for the *in vivo* degradation of sulphatide, meaning that the effect of D169N on the activity of the ASA enzyme is greater than that expected from a simple enzyme assay of total cell extract. The combination of the effect of D169N on ASA activity and targeting is estimated to reduce the activity of *in vivo* ASA to about 35% of normal.

When the D169N and $\Delta F374$ sequence variants were together, the activity of the double mutant (0.34% of normal $\pm$ 0.15%, Figure 5.17) was always slightly higher than that of the deletion in isolation (0.26% $\pm$ 0.16% of normal). It is unclear whether this increase is of significance, but given the fact that in addition, the patient is also homozygous for the ASA-PD allele and would be expected to have very low levels of functional ASA activity, there is little wonder that this patient presented with the severe form of MLD.

The D169N sequence variant may have a significant role to play in the presentation of patient LE who inherited an allele carrying D169N and ASA-PD from her father (KB) and a late infantile mutation, A212V, from her mother. The A212V mutation was identified in the patient by SSCP and sequence analysis, but the allele was subsequently found to have been previously described in an English late-infantile MLD patient (Barth *et al.*, 1994).

The D169N sequence variant is clearly non-pathogenic. This is established by the fact that two normal individuals were identified as double homozygotes for D169N and the ASA-PD allele. Both D169N and N350S affect the targeting of the ASA enzyme to the lysosome. The question of how the D169N/N350S enzyme is targeted to the lysosome is clearly of great

a



b

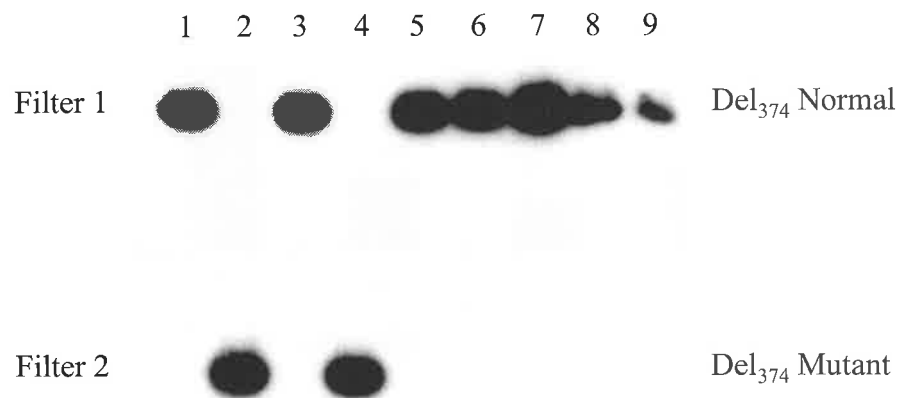
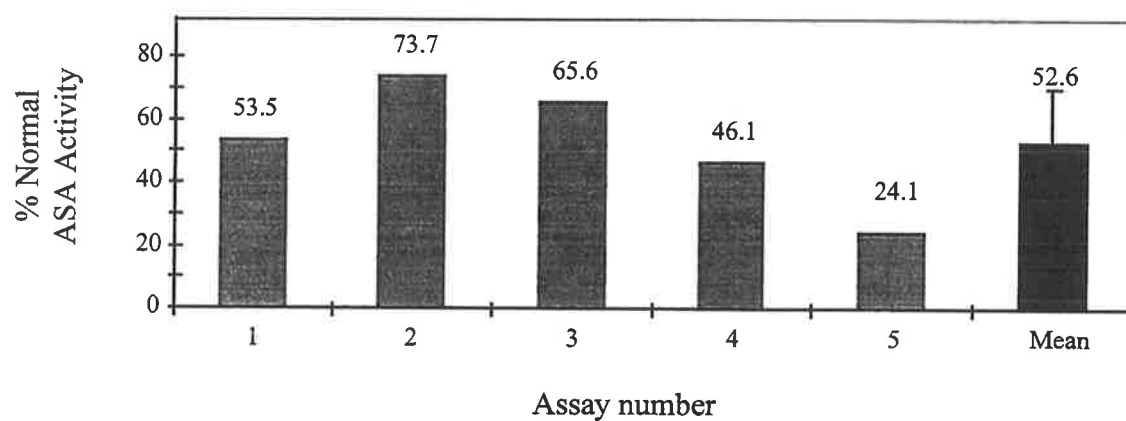


Figure 5.14 Analysis of the D169N and Δ F374 expression constructs

Duplicate filters 1 and 2 contain exon 3/4 (a) and 7/8 (b) amplified cDNA PCR products from the Δ F374 (lane 2), D169N (lane 3) and D169N/ Δ F374 (lane 4) expression constructs. Lanes 1 and 5 to 9 are controls. Filters 1 and 2 in figure a and b were hybridised with the D169N and Δ F374 normal and mutant sequences respectively.

a



b

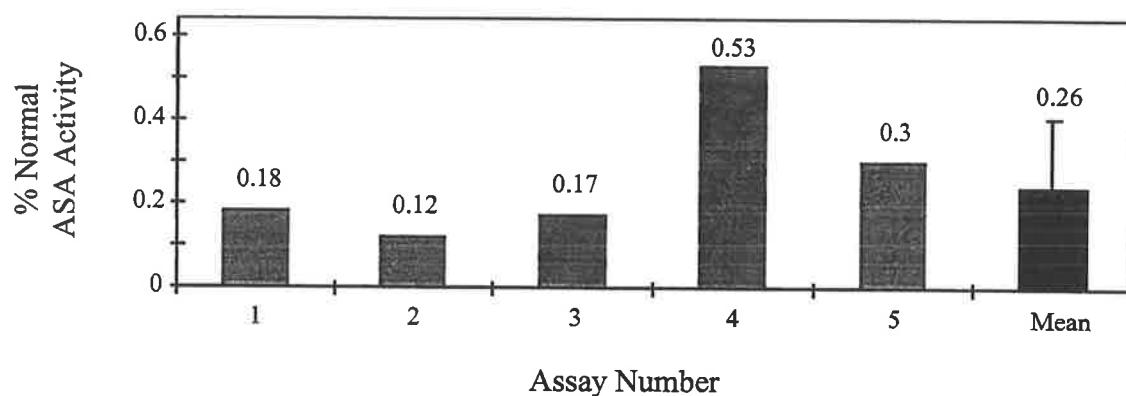


Figure 5.15 Expression of the D169N and $\Delta F374$ sequence variants

Columns 1 to 5 represent the expression of the D169N (a) and $\Delta F374$ (b) mutant constructs relative to that of the normal. The expression of both constructs was measured in 5 quiescent cultures. The mean level of expression is given in the last column of each graph with the respective error bars representing one standard deviation.

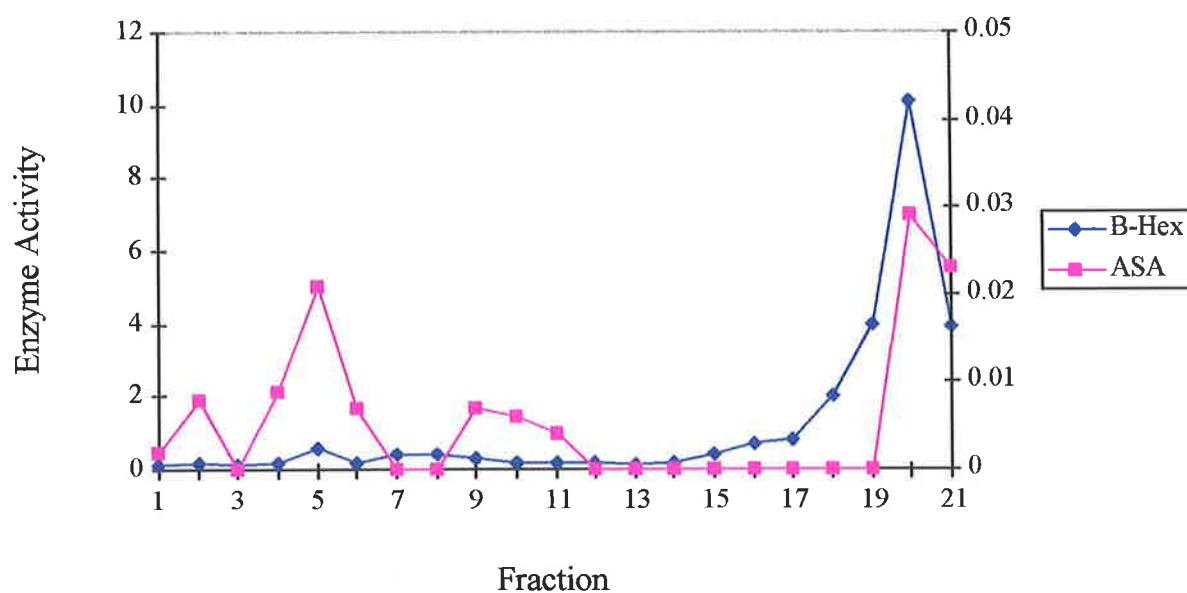


Figure 5.16 Lysosomal targeting of the D169N ASA enzyme

The organelles from a confluent flask of CHO cells transfected with the D169N ASA expression construct were fractionated on a Percoll[®]/sucrose density gradient. Each of the fractions (N=21) were assayed for ASA and β -hexosaminidase activity. The left Y axis represents β -hexosaminidase activity in relative fluorescence units, the right hand Y axis ASA activity in units of absorbance at A_{515} .

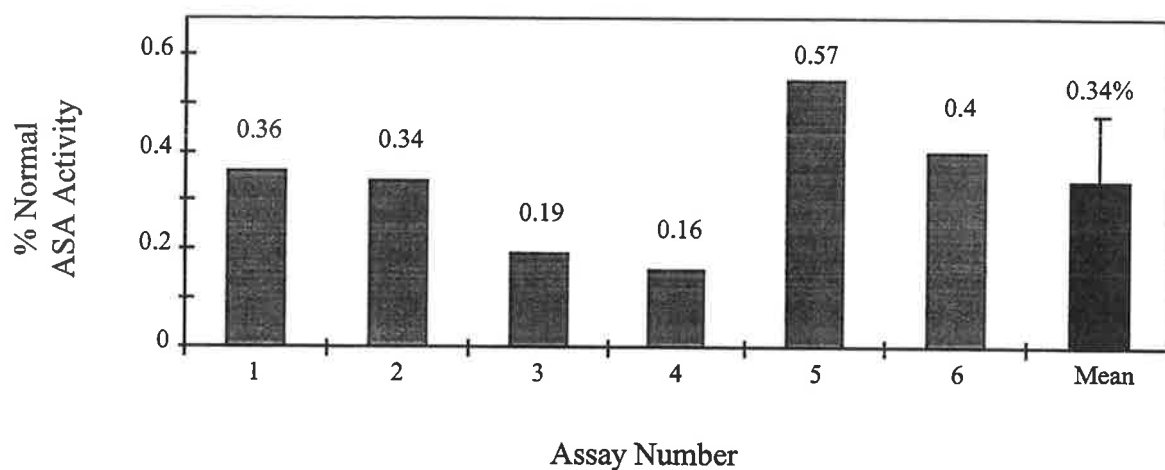


Figure 5.17 Expression of the D169N/ Δ F374 double mutant

The D169N/ Δ F374 construct was assayed in six experiments (columns 1 to 6) where the double mutant construct and the normal were grow synchronously and assayed when the cells were in a quiescent phase of growth. Column seven represents the mean level of relative expression with the error bar showing one standard deviation from the mean.

importance, since the cause of the mistargeting in each case may be exaggerated when both defects are present in the same polypeptide. Regardless of the effect that the two sequence variants have on the activity and targeting of ASA, the end result is a very low, yet non-pathogenic, level of activity.

When the N350S sequence variant was expressed in combination with D169N (Figure 5.18) the level of expression of the D169N allele was reduced from 53% to $25\% \pm 4.2\%$ ($n=3$) of normal (Figure 5.19). This indicates that the reduction of activity caused by each variant in isolation is apparently additive when they are expressed on the same allele.

The effect of D169N and N350S on lysosome targeting does not however, seem to be additive in the same fashion. In fact the distribution of the double mutant ASA activity through the Percoll[®]/sucrose gradient mirrors that of the distribution of the N350S sequence variant alone. There is no evidence of the endosomal bound fraction of ASA in the upper fractions of the gradient as in the D169N Percoll[®]/sucrose fractionation. One explanation for this may be, that there is no difference in the number of glycosylated asparagines in the D169N/N350S and N350S ASA polypeptides and the relative effect of the alteration in glycosylation pattern in each case is similar.

A reduction of *in vivo* ASA activity would be expected however, due to the effect that the D169N and N350S variants have on the targeting of ASA to the lysosome. Although not additive in their effect, only about 50% of the available ASA is correctly targeted (Figure 5.20). The D169N/N350S double homozygote is estimated therefore to express about 12% of normal activity. It must be remembered that in all individuals, such as KB, the D169N/N350S allele also contains the ASA-PD polyadenylation defect which produces only 31% of normal mRNA and would reduce the activity of the ASA-PD/D169N allele to approximately 4% of normal, a level of activity that must be very close to the disease threshold.

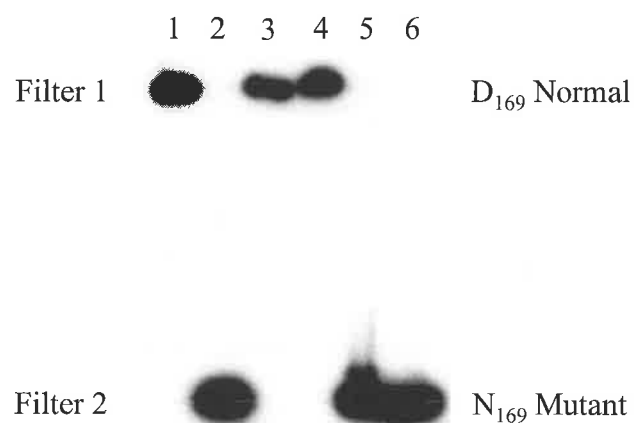


Figure 5.18 Construction of the D169N/N350S double mutant construct

Filters 1 and 2 are duplicates containing exon 3/4 amplified cDNA PCR products hybridised with the normal and mutant D169N ASOs respectively. Lanes 1 and 2 are amplified products from the normal and D169N expression clones respectively, lanes 3 and 4 are P155R/N350S double mutant constructs and lanes 5 and 6 D169N/N350S expression constructs. The presence of the N350S sequence variant within the D169N/N350S constructs was confirmed in Figure 5.11.

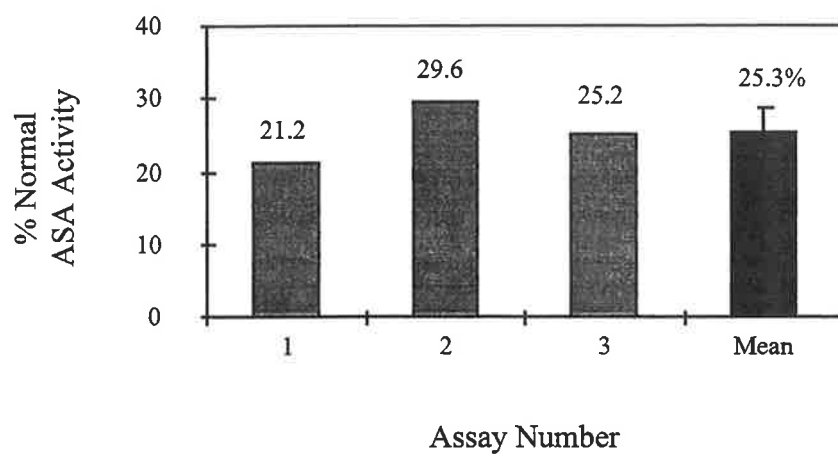


Figure 5.19 Expression of the D169N/N350S construct

The relative level of ASA activity expressed by the D169N/N350S ASA construct was measured in three separate assays of synchronously grown quiescent cells (columns 1 to 3). Column four gives the mean level of relative expression with one standard deviation.

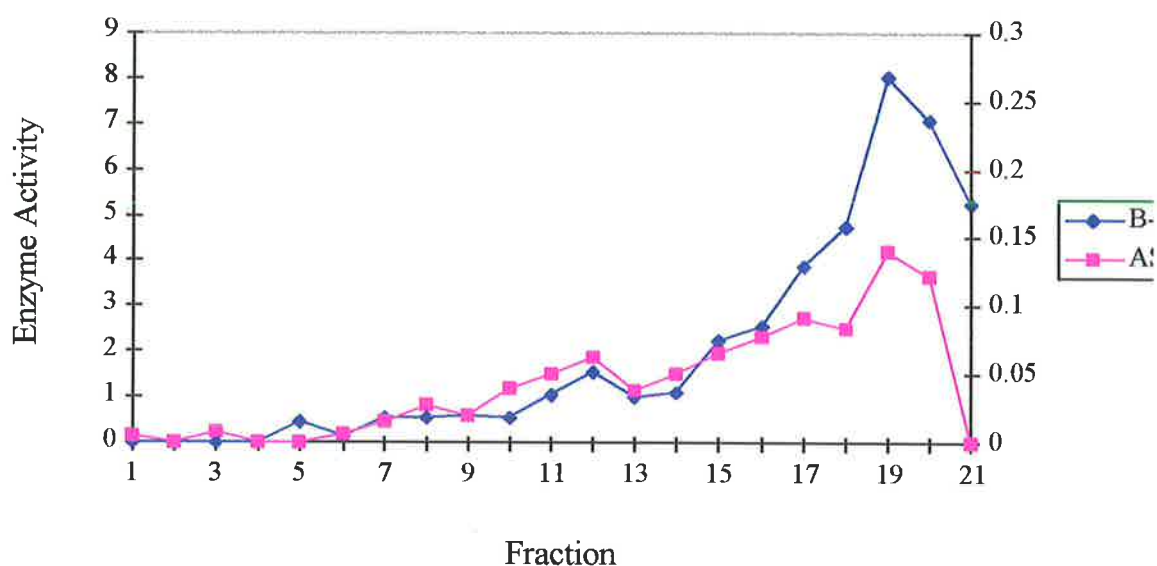


Figure 5.20 Lysosomal targeting of the D169N/N350S ASA protein

Cellular organelles fractionated on a Percoll[®]/sucrose gradient were assayed for β -hexosaminidase and ASA activity.

Units on the left hand Y axis represents β -hexosaminidase activity in relative fluorescence units. The right hand Y axis represents ASA activity measured with the artificial substrate p-nitrocatechol-sulphate in units of absorbance at A_{515} .

The combination of an ASA-PD/D169N allele with a severe late-infantile allele (A212V) in patient LE is expected to produce an *in vivo* level of ASA expression of about 2% of normal. Unfortunately, this level of expression does not account for the presentation of this patient with late-infantile MLD since the most severe MLD patients have basically no detectable ASA activity. The 2% of normal activity produced by a halving of her father's calculated ASA activity would most likely only account for the presentation of the adult form of the disorder. Additionally, it is nonsensical to suggest that the halving of the residual ASA activity found in a normal individual like KB, could lead to the presentation of late-infantile disease, irrespective of the initial level of ASA activity.

It is therefore difficult to explain the presentation of patient LE in view of her father's genotype. The late-infantile form of MLD is usually produced by the inheritance of two severe mutations. The A212V mutation which was maternally inherited has been previously described in a late-infantile patient and therefore accounts for one of these alleles. The paternal ASA-PD/D169N allele is however, clearly not correlated with the late-infantile disease. The homozygous form of this allele expresses 4% of normal activity in KB, if a heterozygous mutation were missed in the SSCP/sequence analysis of this gene then this may account for the inheritance of a paternal infantile allele. Unfortunately this is not a plausible explanation for LE's presentation since if one of the paternal alleles were severe because of an extra unidentified mutation then the residual level of expression in KB would be reduced from its currently estimated 4% of normal to 2%, a level of activity which could be pathogenic. It is also highly likely that the alleles in KB are identical since they appear to be rare, suggesting that KB is homozygous due to a consanguineous union. One can only hypothesize that the unexpectedly severe presentation of patient LE is due to the influence of other unrelated and unidentified, genetic or environmental factors.

Regardless of the explanation for the presentation of this patient, the presence of D169N appears to be having very different effects depending on the background level of ASA activity on which it is expressed. When found in conjunction with the ASA-PD allele it is non-pathogenic although the level of activity which this allele encodes must be close to causing MLD. In contrast, the presence of a single copy of D169N in patient LE is probably the difference between non-pathogenicity and pathogenicity. This hypothesis is supported by the fact that compound heterozygotes for the ASA-PD allele and a severe MLD allele are not affected, yet at this very low level of activity, a small alteration in the expression of the enzyme, (like that produced by the presence of the D169N allele) could have had a great effect on the rate of substrate accumulation and subsequent presentation of disease in this patient.

5.2.3.c Non-pathogenic sequence variants and the presentation of disease

The presentation of patient LP with a complex set of ASA sequence alterations, questions the relative pathogenicity of the different variants, and the way in which they may interact to cause disease. LP was shown to be homozygous for R244C and heterozygous for both the R288C and T391S sequence variants. Each sequence variant and combinations of variants identified in patient LP were introduced into the ASA/M13 clone (data not shown) and the mutant sequences ligated into the ASA expression vector. ASO analysis confirmed the presence of the alterations to the ASA gene sequence within specific expression clones (Figures 5.21 and 5.22).

The expression of the R244C construct demonstrated that this was the sequence variant that was predominantly responsible for the presentation of the patient. The R244C construct expressed only $2.2\% \pm 1.5\%$ of normal ASA activity (Figure 5.23) and therefore R244C could most likely be classified as an adult MLD mutation since it produces a very similar level of ASA activity to the adult, P426L, mutation. The mutation was however,

found in combination with both T391S and R288C which expressed $67\% \pm 14.6\%$ and $19\% \pm 5.8\%$ of normal activity respectively (Figure 5.24). Neither of these sequence variants are therefore likely to be pathogenic in isolation but, hypothetically, they could have the ability to modify the expression of the R244C construct.

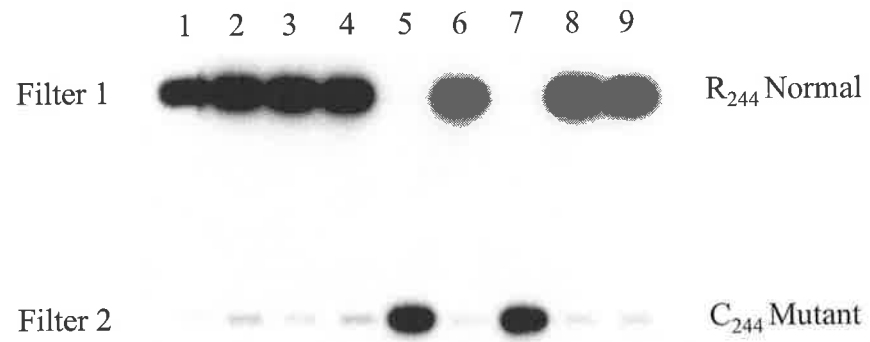
Expression of the double mutant constructs (Figure 5.25) illustrated this effect, T391S reduced the activity of the R244C mutation to $1.7\% \pm 1.5\%$, this is a 23% reduction in activity and approximately mirrors the effect that T391S has on ASA activity in isolation (33% reduction). The R244C/R288C double mutant clone expressed less than 0.5% of normal ASA activity, R288C therefore acts to reduce the level of activity expressed by both the normal and R244C alleles by about 80%. These results indicate that the biochemical effect of the R288C and T391S sequence variants are close to being additive when combined with the R244C mutation.

The R390W mutation was found in patient SA. The results of the expression of this mutation have not been presented because no activity could be detected in CHO cells transfected with the R390W ASA expression construct, relative to the assay of a saline control in two separate assays. In addition to the R390W mutation, a heterozygous SSCP change was identified in exon 6 of patient SA but the cause of this change could not be identified by sequencing exon 6 from either genomic DNA or cDNA from the patient. The late-infantile presentation of patient SA can however be accounted for by the R390W mutation alone.

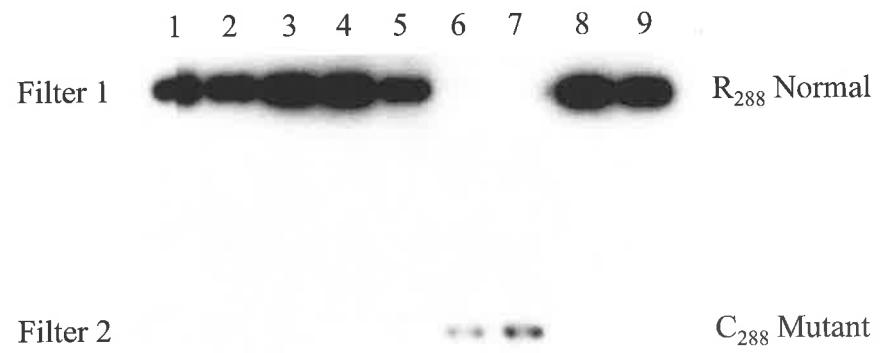
Figure 5.21 ASO analysis of ASA expression constructs

Figures a, b and c are of duplicate filters (1 and 2) which contain exon 3/4, 5/6 and 7/8 cDNA amplified PCR products respectively. All PCR products were amplified from ASA expression constructs. On all filters, lanes 5, 6 and 7 contain amplified products from the R244C, R288C and R244C/R288C expression constructs. Lane 9 contains amplified products from the T391S construct. Lanes 1 to 4 and 8 are controls. The duplicate filters were hybridised with the normal and mutant R244C (a), R288C (b) and T391S (c) ASOs respectively.

a



b



c



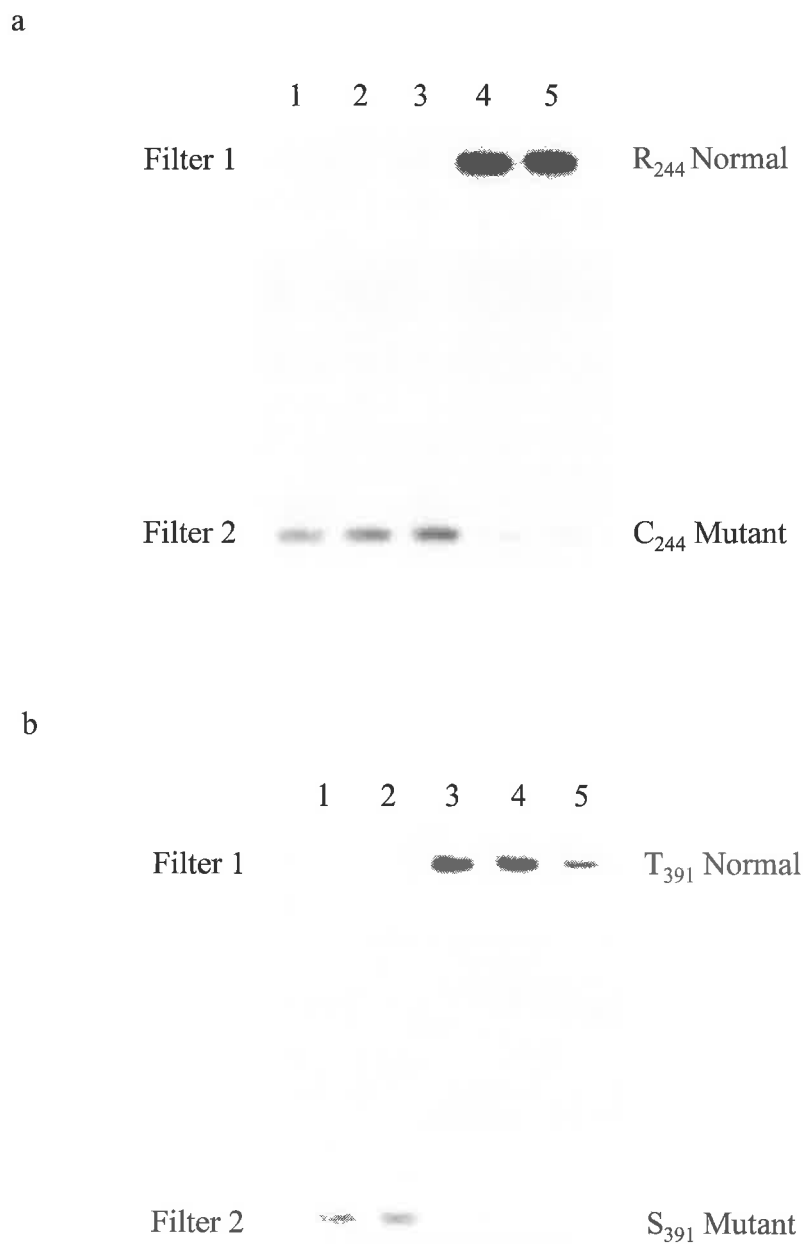


Figure 5.22 Analysis of the R244C/T391S double mutant

a) Filters 1 and 2 in figures a and b are duplicates containing exon 3/4 and 7/8 cDNA PCR products respectively. Lanes 1 to 3 are R244C/T391S expression constructs, lanes 4 and 5 are normal controls. The duplicates have been hybridised with the normal and mutant R244C and T391S ASOs respectively.

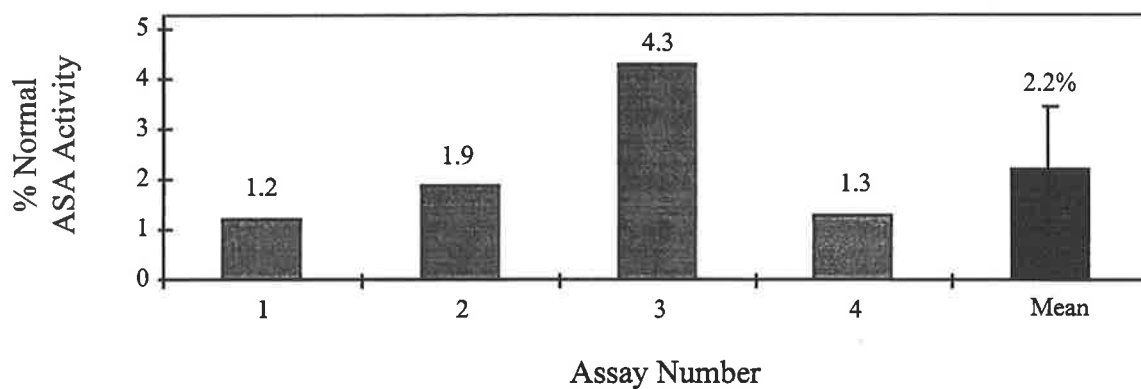
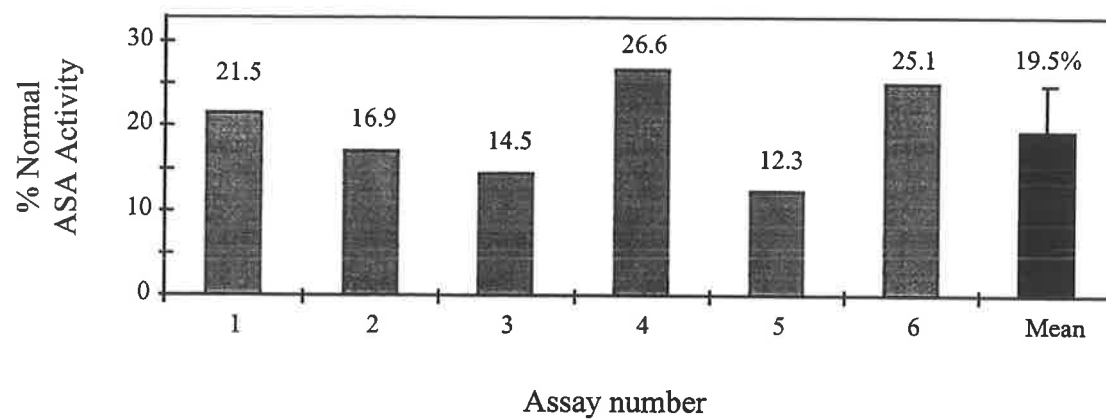


Figure 5.23 Expression of the R244C mutation

The relative activity of the R244C construct was assayed in four sets of confluent quiescent flasks of CHO cells (columns 1 to 4). Column five of the graph represents the mean level of relative expression with one standard deviation.

a



b

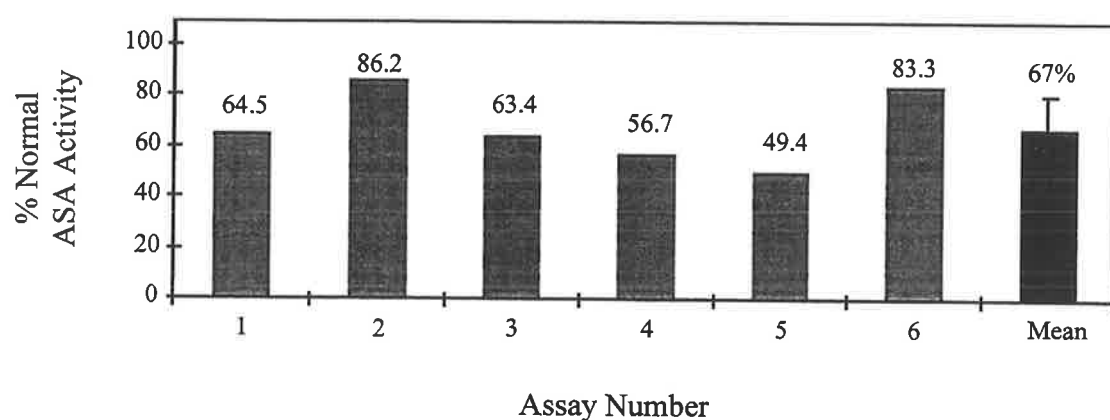
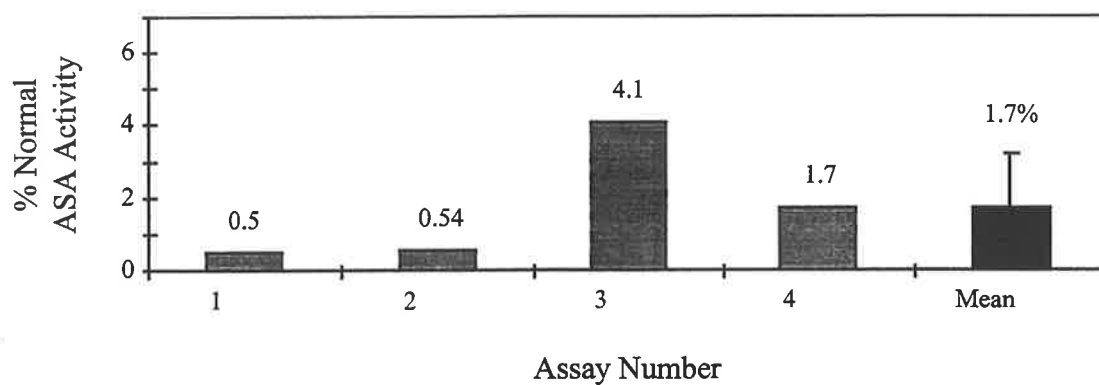


Figure 5.24 Expression of the R244C and T391S alleles

Figures a and b present the results of the expression of the R288C and T391S constructs respectively. The relative activity of both constructs was assayed in six quiescent cultures, the mean level of activity is displayed in the seventh column of each graph as are error bars which correspond to one standard deviation.

a



b

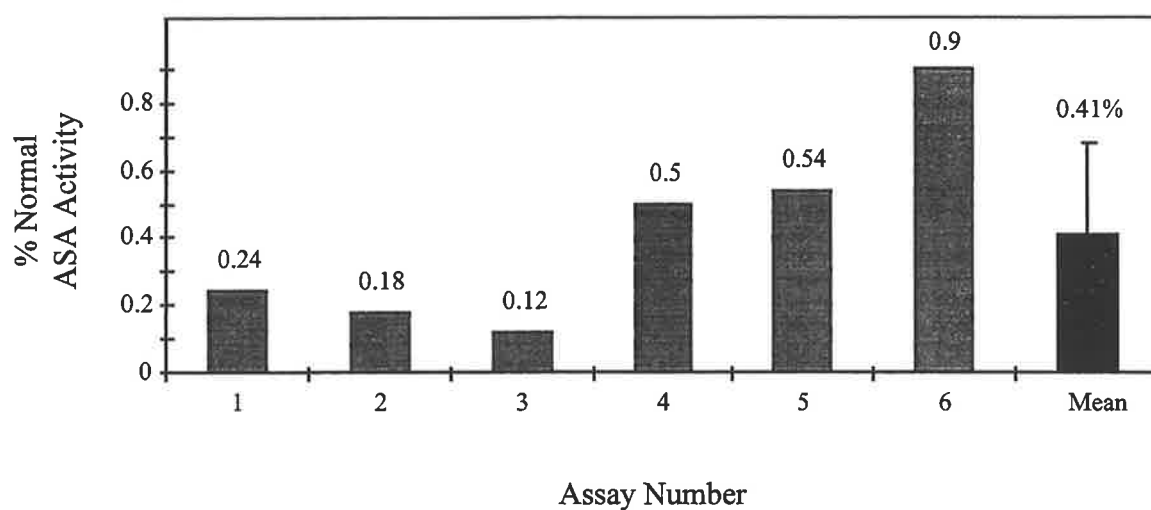


Figure 5.25 Expression of patient LP double mutants

Figures a and b present the expression results of the R244C/T391S and R244C/R288C constructs respectively. The relative activity of each construct was measured in six quiescent cultures of cells, (columns 1 to 6). Column 7 in figure a and b gives the mean level of relative ASA activity with one standard deviation.

5.4 CONCLUSIONS

The establishment of an assay system enabling the comparison of the expression of the normal ASA cDNA with those encoding specific ASA sequence variants has provided insight into the roles that different ASA mutations and particularly, combinations of ASA mutations and sequence variants, can play in the causation of MLD.

Two severe pathogenic mutations were identified in this study. The $\Delta F374$ mutation, identified in patient WT, expressed barely detectable levels of ASA activity. This level of ASA activity was consistent with the presentation and course of disease in this patient. The combination of the $\Delta F374$ mutation with other ASA defects in patient WT, such as the ASA-PD, allele was of little consequence since the patient already had almost undetectable levels of ASA activity and was severely affected by MLD. It was simply not possible to reduce the measured level of activity in individuals such as WT any further.

In contrast to previous reports (Penzein *et al.*, 1993), it would appear, that under certain circumstances, the ASA-PD allele can play a role in the presentation of MLD. This was demonstrated by the analysis of patient MM who was found to be a double homozygote for the ASA-PD and P155R pseudodeficiency sequence variants. The combination of ASA-PD and P155R (which each non-pathogenically reduced ASA activity to approximately 9% of normal) on a single allele, produced a residual level of ASA activity in patient MM, of less than 1% of normal and most probably accounted for the presentation of the patient. Of great importance in this case, is the finding that sequence variants within a gene need not necessarily be pathogenic in their own right to be involved in the presentation of disease. This is particularly relevant to the ASA-PD allele which could conceivably combine with numerous other non-pathogenic sequence variants to cause MLD. The high frequency of the ASA-PD allele in the population ensures that purely by chance, some individuals will be carriers of not only the ASA-PD allele but of other alterations within the ASA coding

sequence. Further careful studies of such individuals will present the opportunity to evaluate interaction between the ASA-PD and other ASA sequence variants.

It is unclear whether the ASA-PD allele plays a similar role in the presentation of patient PG who was found to be a double homozygote for the ASA-PD allele and the sequence variant, G325C. Despite repeated attempts with the standard mutagenesis protocol outlined in this thesis and the use of commercially available mutagenesis kits, the production of a mutant expression construct encoding the G325C allele has not been successful.

Expression of the allele, as well as the quantification of the loss of ASA mRNA due to the effect of the mutation on splicing, would be required to establish the effect of G325C on ASA activity. Whether G325C causes MLD in isolation remains questionable, but it seems plausible that this sequence variant, like P155R, may express a pseudodeficient level of ASA activity. The observation that the patient found to be homozygous for G325C, like MM, did not present with a severe MLD phenotype supports this suggestion. The G325C/ASA-PD allele must retain enough residual ASA activity to avert the onset of the most severe form of MLD.

Non-pathogenic MLD sequence variants have also been shown to have the ability to alter the expression of pathogenic MLD mutations, this suggests that other non-pathogenic sequence variants may also play a role in the modification of disease severity in other MLD patients. The T391S and R288C sequence variants reduced the residual ASA activity expressed by the R244C mutation and in all likelihood altered the presentation of the patient. These results support the suggestion of Leinekugel *et al.* (1992) who hypothesised that within the pathogenic levels of ASA activity identified in MLD patients, very small shifts in the *in vivo* activity of the enzyme could have a great effect on the course of the disease, because the levels of ASA activity separating the clinical MLD subtypes were very small.

The presence of non-pathogenic sequence variants within a pathogenic allele, therefore may have a great influence on the severity of disease. Polymorphic sequence variants, that non-pathogenically reduce ASA activity, may be critical to the presentation of disease when found in combination with MLD mutations or other non-pathogenic sequence variants.

Chapter 6

Concluding Discussion

6.1 OPENING REMARKS

The severity of disease with which MLD patients present is generally dependent on the degree to which ASA is deficient, which is in turn, a function of the severity of the molecular defect which affects the ASA gene. Variation in the severity of MLD is not, however, solely caused by an allelic series of ASA mutations, although this is a common cause of phenotypic diversity which has been observed in MLD and a wide variety of other genetic disorders (reviewed by McKusick, 1994).

The work presented in this study has established that the presentation of patients with MLD can be affected by the interaction of multiple sequence variants within the ASA gene. This observation also has a number of broad implications for our understanding of the mode of inheritance and the presentation of many genetic diseases. Sequence variants within disease genes which have been previously thought to be benign, may actually play an important role in defining the way genetic disease presents and in defining the severity of clinical presentation.

6.1.1 Modifying Sequence Variants

Work presented in this thesis has established that the expression of ASA mutations can be modified by the presence of other, allelic sequence variants. The severity of disease with which a patient presents can be altered as a consequence of this modification.

The residual ASA activities which are responsible for the development of the different clinical MLD subtypes have been estimated in this thesis. The adult mutation P426L, expresses approximately 2.4% of normal activity, a juvenile mutation is estimated to express 1.2% of normal activity and severe late-infantile mutations express barely detectable levels of

ASA. Although these levels of activity had not previously been quantified, it is clear from the observations of numerous authors (Polten *et al.*, 1991; Leinekugel *et al.*, 1992; Gieselmann *et al.*, 1994), that only small differences in residual ASA activity separate the development of the different clinical subtypes of MLD. The inability of enzymatic assays to differentiate between individuals with juvenile and adult MLD provided the initial evidence to support this hypothesis. Careful assays of ASA activity can however, generally differentiate between the late-infantile and later onset subtypes because there is a difference between the undetectable activity which characterises the severe form of the disorder and the low residual activity which is characteristic of the later onset forms (Leinekugel *et al.*, 1992).

The separation of the clinical MLD subtypes by small differences in residual ASA activity explains why normally non-pathogenic reductions in the activity of the ASA enzyme can have such a great effect on patient presentation, when they are expressed in combination with other MLD mutations. A sequence variant that reduced the activity of ASA by half, for example, would under normal circumstances, be non-pathogenic. However, if this sequence variant was found in an allele containing an adult MLD mutation, it would be expected to reduce the level of residual ASA activity produced by the allele and subsequently increase the severity of disease with which a patient presented.

The effect of the R288C and T391S sequence variants on the expression of the R244C mutation in patient LP clearly demonstrated the role that individually non-pathogenic sequence variants could have on residual ASA activity, and therefore disease severity. The level of ASA activity expressed by the R244C mutation was reduced when it was expressed in combination with the T391S and R288C sequence variants.

Generally, common non-pathogenic sequence variants would be expected to substantially contribute to the severity of disease, in the patients where they are found in

combination with pathogenic mutations. If these sequence variants were not present, the severity of the allele would be expected to be reduced and hence the patient presentation would be less severe.

The ASA sequence variants, ASA-PD and T391S, have allele frequencies of 15% and 40% and were found to reduce the activity of ASA to 8% and 65% of normal respectively. In terms of the presentation of patients with MLD, these two sequence variants, because of their prevalence in the patient population, would be expected to make a substantial contribution to the severity of MLD in the patient population, provided that they are not associated with null alleles. A proportion of the T391S variants for example, are associated with the common late-infantile MLD mutation, 459+1G>A and would therefore have no effect on disease presentation. The 459+1G>A mutation produces a non-functional ASA protein so its association with any other variant would have no bearing on the overall ASA activity expressed by the allele.

MLD is not the only disorder which demonstrates the effect that non-pathogenic mutations can have on disease presentation. A further example is presented by Langdown *et al.* (1989) who summarised the influence that the combination of different sequence variants in the β -globin gene could have on the sickling of red blood cells in sickle cell anaemics. The 'sickling' caused by a primary β -globin mutation was modified by the presence of another allelic sequence variant.

Taroni *et al.* (1992) demonstrated that non-pathogenic mutations in the carnitine palmitoyltransferase II (CPTase II) gene could affect the expression of a pathogenic mutation and thereby alter the clinical presentation of patients. Similarly, the expression of a cystic fibrosis transmembrane conductance regulator gene (CFTR) mutation, R117H, can be altered by the presence of a variable intronic polythymidine track which modifies the proportion of

transcripts containing exon nine of the CFTR gene (Chillon *et al.*, 1995; Kieseewetter *et al.*, 1993). In each of these examples, protein function and hence disease presentation is being modified by the presence of sequence variants which when found in isolation, do not cause disease.

Non-pathogenic alterations do not necessarily have to cause amino acid substitutions to play a role in the presentation of disease. The genes involved in the presentation of two forms of autosomal dominant ataxia, spinocerebellar ataxia type 1 (Orr *et al.*, 1993) and Machado-Joseph disease (Kawaguchi *et al.*, 1994) have been identified. In each case, the molecular defect responsible for the presentation of disease is the expansion of an unstable, exonic CAG trinucleotide repeat. There is a strong relationship between the length of the trinucleotide repeat sequence and the severity of disease. The association between the length of the CAG repeat and severity of disease is particularly striking in cases of SCA I (Ranum *et al.*, 1995). Individuals with 38 repeats are normal yet those with 40 are affected.

As previously stated, a threshold level of ASA activity exists above which the clinical symptoms of MLD do not develop. At low, albeit non-pathogenic levels of activity, relatively small reductions of ASA activity below this level can have a great effect on disease presentation because these reductions occur on a low background level of ASA activity. Similarly, in the case of SCA I, a threshold number of 38 CAG repeats has been identified as being non-pathogenic. A small, otherwise non-pathogenic expansion of the CAG repeat by two, is having a great effect on patient presentation because it has occurred on a background of previous repeat expansions.

The involvement of non-pathogenic sequence variants in the definition of the severity of such a wide range of disorders suggests that the presence of such gene defects may be important in determining the severity of a wide variety of genetic diseases. Genetic changes

which have been previously classed as ‘harmless polymorphisms’ may not be as benign as they appear since disease causing mutations are equally as likely to occur on normal alleles as they are in those alleles which contain common, individually non-pathogenic sequence variants. The existence of disease causing mutations on the background of non-pathogenic changes may not only be important in defining disease severity but also in explaining why unrelated individuals with seemingly identical disease causing mutations, present with different clinical symptoms.

6.1.2 Modifying Genes

The activity of an enzyme can also be regulated by the action of modifier genes, when the expression of one gene product, is affected by the expression of another through an interaction of their gene products. (Romeo and McKusick, 1994). These interactions may present clinically when there are reductions in the activity of the modifier gene or of both gene products. This is relevant to the presentation of MLD because mutations in the ASA and the *sap*-precursor genes (review see Sandhoff *et al.*, 1995) can lead to the presentation of MLD. The clinical presentation of a patient with MLD has not yet been attributed to the combined effect of low residual activity of both gene products but, several other examples have been published which illustrate the effect that modifying genes can have on disease presentation, as outlined below.

An allelic series of mutations in the adenomatous polyposis coli (APC) gene are responsible for the presentation of a variety of familial forms of colon cancer. The severity of disease is generally correlated with the severity of mutations present in the APC allele but there is a degree of familial variation in the presentation of colonic cancers (Dietrich *et al.*, 1993). A murine model for human heritable intestinal cancer exists which may provide an

answer to the question of how familial variation in presentation of APC may have arisen. Dietrich *et al.* (1993) reported the identification of a defect in a novel locus which had the ability to modify the number of tumours, or the severity of disease, that developed in mice which were heterozygous for mutations in the murine homologue of the APC gene.

Similarly, Beggs *et al.* (1992) reported a case which exhibited the action of a modifier gene in patients presenting with an unusual form of Duchenne muscular dystrophy (DMD). DMD is caused by mutations in the X-linked dystrophin gene and it is the most common form of X-linked neuromuscular disease. DMD does not usually present before 4 to 5 years of age, yet several patients with dystrophin deficiencies have been reported presenting at birth. This severe presentation of DMD was shown to be caused by hemizyosity for a dystrophin mutation and heterozygosity for a mutation in the gene responsible for the presentation of a related disease, Fukuyama congenital muscular dystrophy (FCMD). A lowering in functional activity of the FCMD gene product has been proposed to increase the severity of the DMD phenotype because of an interaction between the protein products of the two genes. It therefore seems theoretically possible that more subtle sequence variants of the FCMD gene may modify the presentation of DMD.

6.2 PSEUDODEFICIENCY ALLELES AND DISEASE

Wenger and Louie (1991) defined pseudodeficiency alleles, as those causing the measurement of low enzymatic activity in normal individuals such that the measured level of enzyme activity was difficult to differentiate from that measured in presymptomatic late onset patients. This study has shown that alleles causing a pseudodeficiency of ASA, can contain a number of different alterations, apart from the common Poly (A) and N350S sequence variants.

The non-pathogenic sequence variants, ASA-PD and P155R, (which in isolation cause pseudodeficient reductions in the activity of ASA), can combine on the same allele to cause disease. This observation has raised the question of whether non-pathogenic sequence variants in other genes, which cause pseudodeficient reductions in enzyme activity, can play a role in the presentation of other lysosomal storage disorders and other genetic diseases.

Although MLD is a rare disorder, occurring with a frequency of about 1 in 50,000 live births, however, the lysosomal storage disorders as a group, are inherited with a frequency of approximately 1 in 8000 and therefore account for a significant proportion of individuals presenting with genetic disease. Pseudodeficiency alleles that occur commonly in the normal population could collectively, play a significant role in the presentation of patients with lysosomal storage disorders.

The hypothesis that PD alleles may play a significant role in the presentation of the lysosomal storage disorders as a group is supported by the identification by numerous authors, of other lysosomal pseudodeficiency alleles (reviewed by Thomas, 1994). Triggs-Raine *et al.* (1992) identified a β -hexosaminidase (β -hex) pseudodeficiency allele, which contained the sequence variant, R247W. A deficiency of β -hex is responsible for the presentation of Tay-Sachs disease. As is the case with MLD, compound heterozygotes for a Tay-Sachs pseudodeficiency allele containing R247W and a pathogenic mutation are unaffected despite having very low β -hex activities. Tay-Sachs disease is a common disorder among the Jewish population and carrier detection is important for the detection of couples at risk of having affected children.

It is difficult to enzymatically distinguish between carriers of Tay-Sachs mutations and individuals that are heterozygous for a Tay-Sachs PD allele which contains the R247W sequence variant, this is illustrated by the finding that 32% of non-Jewish individuals

identified as Tay-Sachs heterozygotes by enzymatic assay, were in fact heterozygous for the R247W sequence variant and not a Tay-Sachs mutation. Tomczak *et al.* (1993), reported the identification of R247W in 6 of 19 Jewish and 8 of 14 non-Jewish enzyme defined carriers who did not carry any of the 3 common Tay-Sachs mutations, indicating that the R247W sequence variant is common in both Jewish and non-Jewish populations.

A second Tay-Sachs PD allele was identified in the same region of the β -hex coding sequence by Cao *et al.* (1993). The allele, containing the R249W sequence variant was identified in a 42 year old individual who was a compound heterozygote for this novel allele and the known late-onset Tay-Sachs mutation, G269S. The PD allele containing R249W was subsequently identified in 4 of 63 non-Jewish enzyme defined heterozygotes but was not identified in a population screen of 218 Jewish enzyme defined carriers.

When considering the combined frequency of the R247W and R249W β -hex sequence variants, a total of 38% of all non-Jewish and 3% of Jewish enzyme defined carriers are in fact not carriers of Tay-Sachs mutations, but of a Tay-Sachs PD allele. These individuals are therefore at no risk of having a child with the disease. The screening of Tay-Sachs 'heterozygotes' for these common PD alleles would negate the need for unnecessary and confusing enzymatic prenatal diagnosis (Cao *et al.*, 1993).

Vervoot *et al.* (1995) identified a PD allele, containing a D152N sequence variant, in the β -glucuronidase gene which is defective in Sly syndrome or mucopolysaccharidosis type VII (MPS VII). The allele was found in a compound heterozygous mother of a child with MPS VII and a second unrelated individual who presented with an 'atypical' form of MPS VII. The allele was found in only 1 of 100 unrelated normal controls.

The introduction of an extra asparagine residue in the β -glucuronidase polypeptide by D152N was thought to enhance the ability of the enzyme to target itself to the lysosome,

although its overall effect on the activity of the β -glucuronidase enzyme was to reduce its activity to about 27% of normal, primarily by causing an increase in the rate at which the protein was degraded (Vervoot *et al.*, 1995).

Further PD alleles have been identified in the genes encoding α -L-iduronidase (IDUA) (Aranovich *et al.*, 1996) and fumarylacetoacetase (FAH) (Rootwelt *et al.*, 1994) which are the disease genes responsible for the development of MPS I and tyrosinaemia type 1 (HT1), respectively. Although several sibships have been reported to contain MPS I PD alleles, only one PD allele has been characterised at the molecular level and was found to contain a sequence variant which caused an A300T alteration. Enzyme encoded by the A300T allele shows no activity to an artificial substrate, yet the allele does not cause MPS I.

The HT1 PD allele which contains R341W, causes a reduction in the activity of the FAH enzyme to 26-31% of normal. Known HT1 mutations reduce the activity of the FAH enzyme to below 10% of normal. The reduction in activity caused by the R341W is thought to be caused by a reduction in the stability of the protein product and hence an increase in its rate of degradation. In a population screen of 1032 normal individuals the allele was found with a frequency of 2.2%. Although R341W homozygotes and compound heterozygotes for R341W and known disease causing alleles are normal, the authors speculated that the such individuals may be 'prone' to liver disease, which is a symptom of the late onset chronic form of HT1.

The characterisation, in this study, of pseudodeficiency sequence variants in the ASA gene and the identification of other lysosomal and non-lysosomal pseudodeficiency alleles, serves as a warning of the potential diagnostic pitfalls faced when identifying individuals who are thought to be carriers of disease-causing mutations. The *enzymatic* identification of

individuals with carrier levels of enzyme activity, for example, could be indicative of either heterozygosity for a disease mutation or a pseudodeficiency allele.

Conversely, the *molecular* identification of a pseudodeficiency sequence variant is not necessarily diagnostic of an enzymatic pseudodeficiency. Sequence variants which cause pseudodeficient reductions in enzyme activity can play an important role in the development of disease, if an allele contains more than one pseudodeficient sequence variant. This point is well illustrated by the identification in this study, of three individuals who were homozygous for ASA PD alleles but who were also affected by MLD.

6.3 DEFINING NORMAL ENZYMATIC VARIATION

The level of ASA activity which is required to avert the onset of MLD has been estimated in this thesis to be 3% to 4% of normal activity. Any alteration in the ASA gene sequence which acts to reduce the activity of the ASA enzyme to a level which exceeds this activity threshold, would be expected to be tolerated in an evolutionary sense because there would be no selection against the existence of such alterations within the ASA gene.

There is clearly the potential for existence of multiple non-pathogenic sequence variants in any gene, if the normal level of expression of a gene exceeds that which is required by a cell to function normally. I hypothesise that the genes which encode the lysosomal hydrolases are likely to contain a variety of non-pathogenic sequence variants since these genes produce more protein product than that required for the normal hydrolysis of the natural lysosomal substrates. This hypothesis can potentially, be extended to any disease gene, such as tumour suppressor genes, provided that the normally expressed levels of a protein product from such a gene exceeds the metabolic needs of a cell for that gene product. The CFTR gene is another obvious non-lysosomal example of a gene which produces more protein product

than that required for normal cell function, since the presence of a splicing variant in the CFTR gene reduces the total amount of CFTR to approximately 10% of normal without clinical effect.

This study has demonstrated the role that non-pathogenic sequence variants within the ASA gene can play in the presentation of MLD. However, when these non-pathogenic sequence variants (and pathogenic mutations) are found in isolation they must play a role in defining the normal distribution of ASA activities in the population. The measurement of ASA activity in 894 individuals presenting to the Women's and Children's Hospital showed that the enzyme has a skewed distribution of activity around the mean (Figure 6.1).

The degree to which the activity of ASA varies in the population must in part be governed by the extent to which ASA activity can be reduced without causing disease. The lower the threshold level of activity of a protein that is required for normal cellular function, the greater the scope for sequence variants to cause variation in the activity of an enzyme.

The population variation in the activity of ASA and the skewing of the distribution is almost certainly effected by the high carrier frequency of alleles which contain the T391S (Polten *et al.*, 1991) and particularly, the ASA-PD sequence variants (Nelson *et al.*, 1991) which asymptotically reduce ASA activity.

6.4 CLOSING REMARKS

In conclusion, the relative pathogenicity or severity of an allele may be influenced by a variety different intragenic and extragenic factors. Secondary defects need not be pathogenic in their own right to have a profound affect upon disease presentation. The identification of 'modifying sequence variants' in the MLD and other genes, which act to alter the severity of

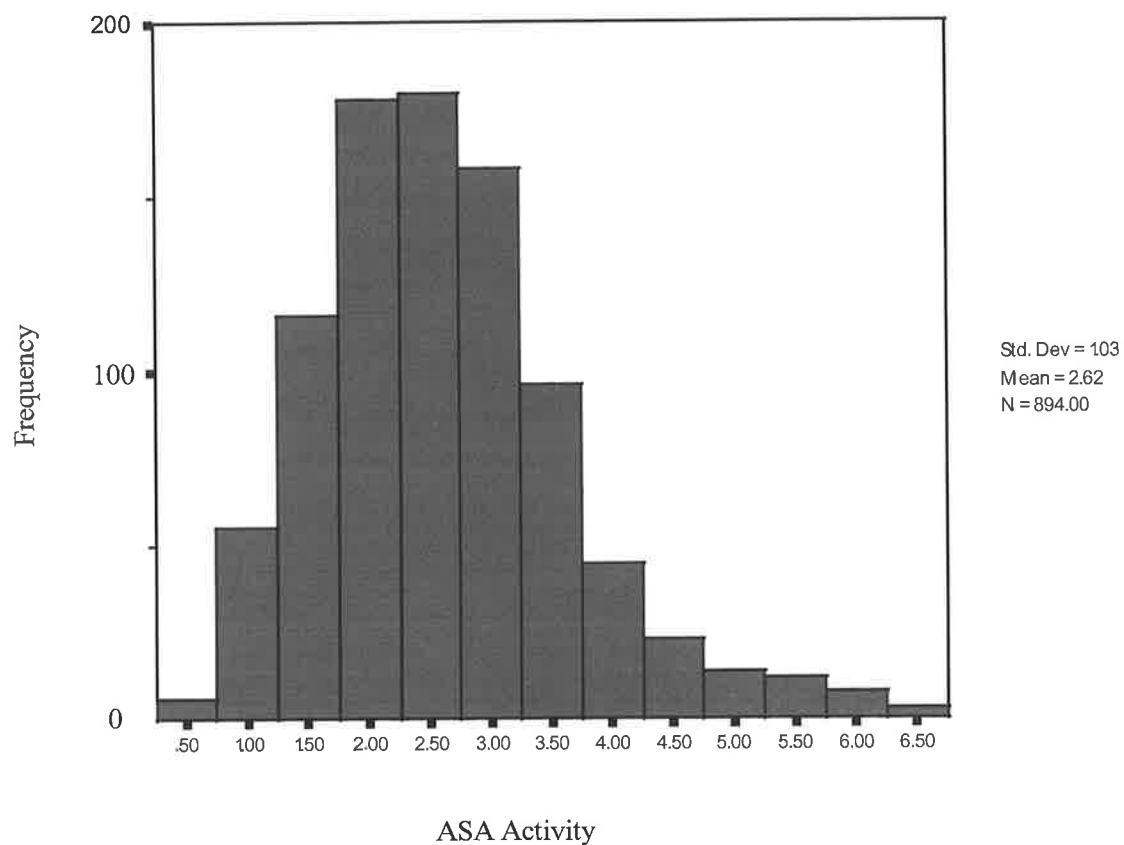


Figure 6.1 Analysis of ASA activity in the normal population.

Histogram representing the distribution of ASA activity measured in the peripheral blood leukocytes of 894 non-MLD individuals presenting to the Women's and Children's hospital. ASA activity was measured using the artificial substrate p-nitrocatechol-sulphate, units of activity are nmol/min/mg of protein.

disease with which patients present, suggests that the presence of non-pathogenic sequence variants within genes could commonly cause modifications in the severity of a wide variety of genetic disorders. This is particularly relevant when the population frequency of some of these non-pathogenic gene variants is considered.

The presence of common non-pathogenic sequence variants on different genetic backgrounds may also be important in the causation of genetic disease and in differentiating the presentation of patients who have seemingly identical pathogenic genotypes, since slight differences in the residual activity of an enzyme can profoundly effect the severity of disease.

6.5 FUTURE WORK

The research presented in this thesis has posed numerous question about the inheritance and development of not only MLD but of other genetic disorders. These questions form the basis of the future lines of inquiry in this field. A number of pathogenic MLD mutations remain to be identified within our patient population and importantly a number of non-pathogenic ASA sequence variants remain to be detected in our population of individuals with pseudodeficient levels of ASA activity.

The identification of non-pathogenic molecular defects is of primary importance since such alterations may be important in defining the severity of disease in the MLD patient population, as demonstrated in this thesis. The fact that 40% of our ASA-pseudodeficiency patients carry only one copy of the common ASA-PD allele suggests that other, as yet unidentified defects in the ASA gene, are responsible for pseudodeficient reductions in the activity of ASA and subsequently the presentation of these individuals with low ASA activities.

The presentation of these individuals with MLD-like neurological symptoms is in itself, puzzling, since their observed clinical symptoms cannot be explained by their low ASA activities. In view of the findings of other authors as to the role of modifier genes in the presentation of disease (see section 6.1.2) it is enticing to hypothesise that their presentation may be due to the combination of their pseudodeficient levels of ASA activity with a defect in a modifier gene *via* an interaction of the two gene products. An obvious place to begin this line of investigation would be to search within the SAP 1 gene for non-pathogenic sequence variants which have the ability to reduce the *in vivo* rate of cerebroside sulphate hydrolysis without effecting the ASA enzyme.

As presented in Chapter 4, a mechanism which acts to regulate the steady state levels of the three ASA mRNAs remains to be characterised. Although I have presented a model to account for the observed frequency of the ASA mRNAs in terms of message stability, this model requires the identification of an 'ASA mRNA regulatory element'. If it can be established that the longer ASA mRNA transcripts are the most abundantly *transcribed* ASA mRNAs this will lend weight to the argument that the stability of the ASA mRNAs is involved in their steady state regulation. The transcription of the ASA mRNAs would be investigated using a nuclear runoff experiment as described in section 4.3.3.

Although numerous ASA sequence variants have been identified and characterised in this study, the mechanism by which each alteration and the combination of alterations in complex alleles, effect the function of the ASA enzyme is unknown. The production of an antibody against the ASA protein would go some way to answering the question of whether sequence variants effect protein stability or catalytic activity. This investigation would allow the identification of important structural elements within the ASA protein and add to our understanding of the role that conserved residues play in the function of other related sulphatase enzymes.

The finding that non-pathogenic sequence variants are not necessarily benign and that they *can* play a role in the development of disease is an important discovery in terms of the way we perceive the inheritance and development of a variety of genetic diseases. The most important lines of investigation arising from this work may not therefore, necessarily be specifically related to MLD but to the role that non-pathogenic sequence variants play in the presentation of other disorders, since as suggested in section 6.3, non-pathogenic sequence variants are likely to be present in a variety of different disease genes.

Appendices

Appendix 1 Allele specific oligonucleotides used to identify previously characterised ASA mutations

Mutation.	Sequence.	Wash Temperature.
R84Q ¹	Normal: 5' CCG GTT CGG ATG GGC AT 3' Mutant: 5' CCG GTT CAG ATG GGC AT 3'	57°C
S96P ²	Normal: 5' CCC CCC GGG AGC TGG GCA 3' Mutant: 5' CCC CCC GGA AGC TGG GCA 3'	63°C
G99D ³	Normal: 5' GGG GGG GCC TGC CCC T 3' Mutant: 5' GGG GGG ACC TGC CCC T 3'	55°C
The G99D mutation was detected by the authors using restriction analysis. The ASOs used in this case were designed during this project.		
459+1G>A ⁴	Normal: 5' TGG TTC CTA TCT GGT CGT 3' Mutant: 5' TGG TTC CTA CCT GGT CGT 3'	57°C
I179S ⁵	Normal: 5' CAA CAG TGG GAT GGG GAC 3' Mutant: 5' CAA CAG TGG GCT GGG GAC 3'	56°C
N350S ⁶	Normal: 5' AA GGT GAC ATT GGG CAG TGG 3' Mutant: 5' AA GGT GAC ACT GGG CAG TGG 3' Mutant: 5' CTG CCC AGT GTC ACC T 3' ^{##}	60°C
T391S ⁴	Normal: 5' GTG CGG ACT GGA AAG TA 3' Mutant: 5' GTG CGG AGT GGA AAG TA 3'	61°C

P426L⁴ Normal: 5' AG CCC CCG CTG CTC TA 3' 59°C

Mutant: 5' AG CCC CTG CTG CTC TA 3'

1401+1G>A⁵ Normal: 5' AGG GGT TAC CCT GGG TGA 3' 61°C

Mutant: 5' AGG GGT TAT CCT GGG TGA 3'

1204+1G>A⁷ Normal: 5' GTC ACA GCT GCG TCT AA 3' 45°C

Mutant: 5' CCC AGT TAG ACC TTC CGG 3'

ASA-PD⁶ Normal: 5' CTG GTG TTA TTA CGT TAT C 3' 52°C

Mutant: 5' CTG GTG TTA CTA CGT TAT C 3'

¹ Kappler *et al.*, (1992). ² Gieselmann, V. *et al.*, (1991). ³ Kondo *et al.*, (1991). ⁴ Polten *et al.*, (1991). ⁵ Fluharty *et al.*, (1991). ⁶ Gieselmann, *et al.*, (1989). ⁷ Bohne, W. *et al.*, (1991).

^{##} used specifically for the introduction of the S350 mutation into the M13 ASA cDNA clone.

Appendix 2 Oligonucleotides used to amplify the genomic copy of the ASA gene

Exon	Primer	Sequence	Annealing time/temp
1	Ex1A	5' GAG ACA GAC AGA AAT GTG GCC TCC 3'	60°C/30sec
	ON1*	5' TGC TGG AGC CAA GTA GCC CT 3'	
2	Ex2B	5' CCC TTT CCT CCC AAT GGA TTG CAG 3'	57°C/40sec
	Ex2C	5' GAGGGTGGCTGAGGGCCCCGGGTGG 3'	
3	ON1A	5' CAT CGA TTT CTA GGC ATC CC 3'	61°C/40sec
	ON3	5' CAA AGA CTG GAG TTA GCA CT 3'	
4	Ex4D	5' CTC TCA CGT AAG TGA TCT TGG CCC 3'	57°C/40sec
	Ex4C	5' AAG CAG CTG AAC TGC AAG GCC TCC 3'	
5/6	Ex5/6F	5' CAT GGC TCA TGA GCG CCT CTG TGT 3'	55°C/30sec
	Ex5/6G	5' GGG GCC AAG GAT CTG GAA TCA GGG 3'	
	Ex5Rev	5' GTG GCC AGC TCG TGG GTC ACG CCT 3'	
	ON16	5' TTG ATG GCG AAC TGA GTG A 3'	
7	Ex7H	5' GCT GCT GGG CAC AGG CAA GGT AGG 3'	57°C/40sec
	Ex7I	5' TAG GGA GGG GGG CTT GGA CGA CT 3'	
8	Ex8J	5' ACA CTA CCA GGA GTG ACC CTC AAC 3'	57°C/40sec
	ON16B	5' TCG GCA GTC TCT CTT CTC CT 3'	

All reaction were performed on a Perkin Elmer Cetus thermo cycler with an initial denaturation for 7 min at 95°C, followed by 34 cycles of denaturation at 95°C for 30 sec, annealing at various times and temperatures as above and extension at 72°C for 45 sec. This was followed by a final extension at 72°C for 3 min. * primers designed by Polten *et al.* (1991).

Appendix 3 Amplification of the ASA cDNA.

Exon	Primer	Sequence	Annealing time/temp
1/2	ON1*	5' TGC TGG AGC CAA GTA GCC CT 3'	58°C/40sec
	ON3A	5' GAA GCA GGT CAG GTT CTG GCA G 3'	
3/4	Ex3/4 For	5' CAG GGC TTC CAT CGA TTT CTA GGC 3'	60°C/40sec
	Ex3/4 Rev	5' CCT CGG GAC ATA CGC ATG GTC TCA 3'	
5/6	Ex5/6 For	5' CTG CTT GAA GAG ACG CTG GTC ATC 3'	68°C/40sec
	Ex5/6 Rev	5' AGG CAG GGT CTG CAG TGG TAT CAC 3'	
7/8	Ex7/8 For	5' AAG TAC AAG GCT CAC TTC TTC ACC 3'	58°C/40sec
	Ex7/8 Rev	5' GGC TCC CAG TGA GGA GCC ATC ACA 3'	

*Polten *et al.*, (1991).

All cDNA PCR retains began with an initial denaturation for 2min at 93°C, followed by 32 cycles of denaturation at 93°C for 30 sec, annealing time and temperature as above for the respective amplifications and 72°C extension for 45 sec. This was followed by a final extension of 72°C for 2 min. The exon 7/8 reaction went through 34 cycles of amplification.

Appendix 4 Allele specific oligonucleotides used to detect novel sequence variants

Exon.	ASO.		Sequence.	Critical wash temperature.
3	P155R	Normal	5' CAG GGC CCC TGC CAG A 3'*	n/a
		Mutant	5' CAG GGC CGC TGC CAG A 3'*	
		Normal	5' CT CAG GGC CCC TGC CAG 3'	60°C
		Mutant	5' CT CAG GGC CGC TGC CAG 3'	
3	D169N	Normal	5' ACT CCT TGC GAC GGT GG 3'	60°C
		Mutant	5' ACT CCT TGC AAC GGT GG 3'	
4	R244C	Normal	5' GT TCA GGC CGC GGG CC 3'	66°C
		Mutant	5' GT TCA GGC TGC GGG CC 3'	
4	T274M	Normal	5' TT GAA GAG ACG CTG GTC A 3'	62°C
		Mutant	5' TT GAA GAG ATG CTG GTC A 3'	
5	R288C	Normal	5' AGA CCA TGC GTA TGT CC 3'	64°C
		Mutant	5' AGA CCA TGT GTA TGT CC 3'	
5	G325C	Normal	5' C GCT CCC GGC GTG ACC 3'*	n/a
		Mutant	5' C GCT CCC AGC GTG ACC 3'*	
		Normal	5' C GCT CCC GGT CAG TCC G 3'	64°C
		Mutant	5' C GCT CCC AGT CAG TCC G 3'	
7	Del374	Normal	5' GTC TCT CTT CTT CTA CCC 3'	57°C
		Mutant	5' CA GTC TCT CTT CTA CCC G 3'	

7	R390W	Normal	5' TT TGC TGT GCG GAC TGG A 3'	58°C
		Mutant	5' TT TGC TGT GTG GAC TGG A 3'	
8	Q486X	Normal	5' C GCC CTG CAG ATC TGC TG 3'	59°C
		Mutant	5' C GCC CTG TAG ATC TGC TG 3'	

* Primers designed to hybridise to the ASA cDNA sequence. n/a: not applicable, primers were not used in a population ASO screen.

Appendix 5 Phosphor-imaging quantification of protected products of RBNP gels

Ribonuclease Protection

Assays.

All values are measures of the integral volume of bands in pixels.

Gel 1

		2.1	3.7+4.8	GAPDH	Phenotype
Lane 1	#	80989	49174	710877	Normal
Lane 2	#	293566	145270	3997735	Normal
Lane 3	#	204631	123709	2227760	Normal
Lane 4	upper control	60760	135302	4781035	Pseudodeficient
	Band	63441			
	lower control	57472			
Lane 5	upper control	57954	247325	4149530	Pseudodeficient
	Band	63850			
	lower control	63525			

Gel 2

Lane 1	#	178498	121760	1803936	Normal
Lane 2	#	149727	95228	1570506	Normal
Lane 3	upper control	23585	129674	1566741	Pseudodeficient
	Band	24191			
	lower control	23498			
Lane 4	upper control	55343	45601	693497	Pseudodeficient
	Band	56207			
	lower control	51859			

Gel 3

Lane 1					
Lane 2	#	85898	56822	1676458	Normal
Lane 3	#	66852	50740	902478	Normal
	upper control	14615	41825	441463	Pseudodeficient
	Band	14618			
	lower control	13718			

The protected 2.1 kb fragments contains 29 labelled T's the 3.7 and 4.8 kb fragments contain 43.

Appendix 6 ASA activity in exponentially growing CHO cells containing the normal and P155R expression constructs

Raw data showing the expression of the normal and P155R ASA expression constructs. ASA assays were performed with the artificial substrate p-nitrocatechol-sulphate on cells undergoing exponential growth. All cell lines were harvested and assayed approximately 24 hrs pre-confluency, 2 days after the cells had been subcultured at a split ratio of 1 to 3.

	Protein Concentration ug/ul		ASA Activity nmol/min/mg of protein		
<u>Assay</u>	<u>Normal</u>	<u>P155R</u>	<u>Normal</u>	<u>P155R</u>	<u>% Normal Activity</u>
1	3200	4160	399.8	19.7	4.9
2	4160	1360	363.1	60.5	16.6
3	1040	1440	255.3	23.6	9.3
4	1280	800	494.3	70.6	14.3
5	3040	2680	220.3	37.8	17.7
6	2560	1960	445.9	52.2	11.7
7	1540	1220	553.8	34.5	6.2
8	620	960	671.3	35.9	5.3
9	1860	1620	505.2	20.8	4.1
10	4240	4040	614	32	5.4
11	4200	2680	524.5	17.9	2.8
12	2160	1280	290.2	17.7	6.1
				Mean	8.7% ± 4.5%

Appendix 7 Normal and P155R assays using the natural ASA substrate

Of the twelve cultures of cells assayed in Appendix 5.1, eight were concurrently assayed for ASA activity using the natural ASA substrate, sulphatide. Protein concentration for the various cultures are presented in the corresponding assay in Appendix 5.1

All units of activity are in nmol/min/mg of protein for both the normal and P155R assays. The activity of the P155R construct is also expressed as a proportion of the normal.

<u>Sulfatidase Activity</u>			
<u>nmol/min/mg</u>			
<u>of protein</u>			
<u>Assay</u>	<u>Normal</u>	<u>P155R</u>	<u>% Normal Activity</u>
1	na	na	na
2	15723.3	2154.6	12.1
3	10317.7	395.1	3.6
4	na	na	na
5	na	na	na
6	12258.7	781.7	7.04
7	na	na	na
8	6819.2	185.3	2.6
9	6399.2	91.2	1.4
10	8187.3	411.2	4.8
11	13978.2	198.1	1.4
12	7479.6	917.7	10.9
		Mean	5.5% $\pm$ 3.5%

na: denotes the cultures which were not assayed with the natural substrate.

Appendix 8 ASA assay of confluent quiescent CHO cells

The P155R and normal ASA expression constructs were assayed in CHO cells which had reached confluency and allowed to enter a quiescent stage of growth for 3 days. The results presented here are from seven assays of the normal and P155R expression lines. Units of enzyme activity are in nmol/min/mg of protein. Protein concentrations are in $\mu\text{g/ml}$. The expression of the P155R construct has been calculated as a percentage of that measured in the normal construct.

Protein Concentration $\mu\text{g}/\mu\text{l}$			ASA Activity nmol/min/mg of protein		
<u>Assay</u>	<u>A35</u>	<u>P155R</u>	<u>Normal</u>	<u>P155R</u>	<u>% Normal Activity</u>
1	1800	1650	254.3	22.2	8.7
2	1800	1650	197.9	20.6	10.4
3	1960	1400	805.2	67.4	8.4
4	2480	2200	372.3	26.3	7.1
5	1400	1440	453	39.9	8.8
6	1600	1560	282.1	36.5	12.9
7	1760	2080	432.2	42.1	9.7
				Mean	$9.4\% \pm 1.8\%$

PUBLICATIONS AND AWARDS

The results presented in this thesis have been published or submitted for publication in the following papers:

Harvey, J. S., Nelson, P. V., Carey, W. F., Robertson, E. F. and Morris, C. P. (1993). An arylsulphatase A (ARSA) missense mutation (T274M) causing late-infantile metachromatic leukodystrophy. *Human Mutation* **2**: 261-267.

Harvey, J. S., Carey, W. F., Nelson, P. V. and Morris, C. P. (1994). Metachromatic Leukodystrophy: a nonsense mutation (Q486X) in the arylsulphatase A gene. *Human Molecular Genetics*. **3**: 207.

Crawford, J., Labrinidis, A., Carey, W. F., Nelson, P. V., Harvey, J. S. and Morris, C. P. (1995). A splicing mutation (1898 + 1G>T) in the CFTR gene causing cystic fibrosis. *Human Mutation* **5**: 101-102.

Woollatt, E., Harvey, J. S., Callen, D. F., Carey, W. F. and Morris, C. P. (1996). Arylsulphatase A lysosomal enzyme map position 22q13.3. *Chromosome Research*. **4**: 407.

Harvey, J. S., Carey, W. F. and Morris, C. P. (1996). Redefining the arylsulphatase A pseudodeficiency allele. Submitted to the *American Journal of Human Genetics*

Harvey, J. S., Carey, W. F. and Morris, C. P. (1996). Metachromatic leukodystrophy caused by the combined affect of two arylsulphatase A pseudodeficiency alleles. Submitted to *Human Molecular Genetics*.

The presentation of the work contained in this thesis has led to the presentation of the following student awards.

1) The award for the best Ph. D. student poster presentation at the 41st annual meeting of the Genetics Society of Australia, at the University of New England, Armidale, NSW. July, 1994.

Title of poster presentation. *Detection and characterisation of mutations causing metachromatic leukodystrophy.*

2) Promega/Lorne Genome Conference Ph. D. student prize presented at the 18th annual conference on The Organisation and Expression of the Genome, February, 1996.

Title of oral presentation: *The role of non-pathogenic gene variants in the causation of genetic disease.*

3) The award for the best Ph. D. student oral presentation at the 20th annual scientific meeting of the Human Genetics Society of Australia, Adelaide, South Australia, September, 1996.

Title of oral presentation: *The role of non-pathogenic gene variants in the causation of genetic disease.*

REFERENCES

- Aranovich, E. L., Pan, D. and Whitley, C. B.** (1996). Molecular genetic defect underlying α -L-iduronidase pseudodeficiency. *American Journal of Human Genetics*. **58**: 75-85.
- Amaducci, L., Sorbi, S., Piacentini, S. and Bick, K. L.** (1991). The first Alzheimer disease case: a metachromatic leukodystrophy ? *Developmental Neuroscience*. **13**: 186-187.
- Austin, J.** (1959). Metachromatic cerebroside sulphates in cerebral white matter and kidney. *Proceedings of the Society of Experimental Biology and Medicine*. **100**: 361-364.
- Austin, J., Balasubramania, A. S., Pattabiraman, T. N., Saraswathi, S., Basu, D. K. and Bachhawat, B. K.** (1963). A controlled study of enzymatic activities in three human disorders of glycolipid metabolism. *Journal of Neurochemistry*. **10**: 805-816.
- Bardosi, A., Friede, R. L., Ropte, S. and Goebel, H. H.** (1987). A morphometric study on sural nerves in metachromatic leukodystrophy. *Brain*. **110**: 683-694.
- Baquis, G. D., Kelly, J. J., Lieberman, A. and Wolpert, S. M.** (1991). Adult metachromatic leukodystrophy and pes cavus foot deformity. *Muscle and Nerve*. **14**: 784-785.
- Barriocanal, J. G., Bonifacino, J. S., Yuan, L. and Sandoval, I. V.** (1986). Biosynthesis, glycosylation, movement through the golgi system, and transport to lysosomes by an *N*-linked carbohydrate-independent mechanism of three lysosomal integral membrane proteins. *Journal of Biological Chemistry*. **261**: 16755-16763.
- Baum, H., Dogson, K. S. and Spencer, B.** (1959). The assay of arylsulphatase A and B in human urine. *Clinica Chimica Acta*. **4**: 453-455.
- Barth, M. L., Fensom, A. and Harris, A.** (1993). Missense mutations in the arylsulphatase A of metachromatic leukodystrophy patients. *Human Molecular Genetics*. **2**: 2117-2121.

- Barth, M. L., Ward, C., Harris, A., Saad, A. and Fensom, N.** (1994). Frequency of arylsulphatase A pseudodeficiency associated mutations in a healthy population. *Journal of Medical Genetics*. **31**: 667-671.
- Beggs, A. H., Neumann, P. E., Arahata, K., Arikawa, E., Nonaka, I., Anderson, M.-D. S. and Kunkel, L. M.** (1992). Possible influences on the expression of X chromosome linked dystrophin abnormalities by heterozygosity for autosomal recessive Fukuyama congenital muscular dystrophy. *Proceedings of the National Academy of Science*. **89**: 623-627.
- Beutler, E. and Grabowski, G. A.** (1995). Gaucher disease. In *The Metabolic and Molecular Basis of Inherited Disease*. 7th Edition, McGraw-Hill, Inc. Health professionals division. Edited by Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D.
- Bohne, W., von Figura, K. and Gieselmann, V.** (1991). An 11-bp deletion in the arylsulphatase A gene of a patient with late infantile metachromatic leukodystrophy. *Human Genetics*. **87**: 155-158.
- Bou-Gharios, G., Adams, G., Pace, P., Warden, P. and Olsen, I.** (1993). Correlation of a genetic deficiency by contact-mediated lysosomal enzyme transfer from bone marrow cells *in vivo*. *Transplantation*. **56**: 991-996.
- Brooks, D. A., Harper, G. S., Gibson, G. J., Ashton, L. J., Taylor, J. A., McCourt, P. A. G., Freeman, C., Clements, P. R., Hoffmann, J. W. and Hopwood, J. J.** (1991). Hurler syndrome: a patient with abnormally high levels of α -L-iduronidase protein. *Biochemical and Biomedical Biology*. **47**: 211-220.
- Butterworth, J., Broadhead, D. M. and Keay, A. J.** (1978). Low arylsulphatase A activity in a family without metachromatic leukodystrophy. *Clinical Genetics*. **14**: 213-218.

- Caillaud, C., Blanchot, C., Akli, S., Crosnier, J. M., Puech, J. P., Kahn, A. and Poenaru, L.** (1993). Molecular basis of late-infantile metachromatic leukodystrophy in France. *In ESGLD workshop, Delphi, Greece, October 1993 (abstract).*
- Cao, Z., Natowitz, M. R., Kaback, M. M., Lim-Steele, J. S. T., Prenc, E. M., Brown, D., Chabot, T. and Triggs-Raine, B. L.** (1993). A second mutation associated with apparent β -hexosaminidase A pseudodeficiency: identification and frequency estimation. *American Journal of Human Genetics.* **53**: 1198-1205.
- Carey, W. F. and Pollard, A. C.** (1977). Variability of fibroblast lysosomal acid hydrolases with reference to the detection of enzyme deficiencies. *Australian Journal of Experimental Biology and Medical Science.* **55**: 245-252.
- Chabás, A., Castellví, S., Bayés, M., Balcells, S., Grinberg, D., Vilageliu, L. I., Marfany, G., Lissens, W. and González-Duarte, R.** (1993). Frequency of the arylsulphatase A pseudodeficiency allele in the Spanish population. *Clinical Genetics.* **44**: 320-323.
- Chabot, T. and Triggs-Raine, B. L.** (1993). A second mutation associated with apparent β -hexosaminidase A pseudodeficiency: identification and frequency estimation. *American Journal of Human Genetics.* **53**: 1198-1205.
- Chaconas, G. and van de Sande, J. H.** (1980). 5' ^{32}P labeling of RNA and DNA restriction fragments. *Methods in Enzymology.* **65**: 75-85.
- Chang, P. L. and Davidson, R. G.** (1983). Pseudo arylsulphatase A deficiency in healthy individuals: genetic and biochemical relationship to metachromatic leukodystrophy. *Proceedings of the National Academy of Science. USA.* **80**: 7323-7327.
- Chao, H. H.-J., Waheed, A., Pohlmann, R., Hille, A. and von Figura, K.** (1990). Mannose 6-phosphate receptor dependent secretion of lysosomal enzymes. *The EMBO Journal.* **9**: 3507-3513.

- Chillón, M., Casals, T., Mercier, B., Bassas, L., Lissens, W., Silber, S., Romey, M.-C., Ruiz-Romero, J., Verlingue, C., Claustres, M., Nunes, V., Férec, C. and Estivill, X.** (1995). Mutations in the cystic fibrosis gene in patients with congenital absence of the vas deferens. *New England Journal of Medicine*. **332**: 1475-1480.
- Chomczynski, P. and Sacchi, N.** (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry*. **162**: 156-159.
- Collins, J. E., Cole, C. G., Smink, L. J., Garrett, C. L., Leversha, M. A., Sonderlund, C. A., Maslen, G. L., Everett, L. A., Rice, K. M., Coffey, A. J., Gregory, S. G., Gwilliam, R., Dunham, A., Davies, A. F., Hassock, S., Todd, C. M., Lehrach, H., Hulsebos, T. J. M., Weissenbach, J., Morrow, B., Kucherlapati, R. S., Wadey, R., Scambler, P. J., Kim, U.-J., Simon, M. I., Peyrard, M., Xie, Y.-G., Carter, N. P., Durbin, R., Dumanski, J. P., Bentley, D. R. and Dunham, I.** (1995). A high density YAC contig map of human chromosome 22. *Nature*. **377** (6547 Suppl): 367-379.
- Conzelmann, E. and Sandhoff, K.** (1983/84). Partial enzyme deficiencies: residual activities and the development of neurological disorders. *Developmental Neuroscience*. **6**: 58-71.
- Davis, L. E., Snyder, R. D., Orth, D. N., Nicholson, W. E., Kornfeld, M. and Seelinger, D. F.** (1979). Adrenoleukodystrophy and adrenomyeloneuropathy associated with partial adrenal insufficiency in three generations of a kindred. *American Journal of Medicine*. **66**: 342-347.
- De Hostos, E. L., Schilling, J, Grossman, A. R.** (1989). Structure and expression of the gene encoding the periplasmic arylsulphatase of *Chlamydomonas reinhardtii*. *Molecular and General Genetics*. **218**: 229-239.

Dietrich, W. F., Lander, E. S., Smith, J. S., Moser, A.S., Gould, K. A., Luongo, C., Borenstein, N. and Dove W. (1993). Genetic identification of *Mom-1*, a major modifier locus affecting *Min*-induced intestinal neoplasia in the mouse. *Cell*. **75**: 631-639.

Dubois, G., Turpin, J.-C. and Baumann, N. (1975). Absence of ASA activity in healthy father of a patient with metachromatic leukodystrophy. *New England Journal of Medicine*. **293**: 302.

Dubois, G., Harzer, K. and Baumann, N. (1977). Very low arylsulphatase A and cerebroside sulfatase activities in leukocytes of health members of metachromatic leukodystrophy family. *American Journal of Human Genetics*. **29**: 191-194.

Fluharty, A. L., Meek, W. E. and Kihara, H. (1983). Pseudo arylsulphatase A deficiency: evidence for a structurally altered enzyme. *Biochemical and Biophysical Research Communications*. **112**: 191-197.

Fluharty, A. L., Fluharty, C. B., Bohne, W., von Figura, K. and Gieselmann, V. (1991). Two new arylsulphatase A (ARSA) mutations in a juvenile metachromatic leukodystrophy patient. *The American Journal of Human Genetics*. **49**: 1340-1350.

Francis, G. S., Bonni, A., Shen, N., Hechtman, P., Yamut, B., Carpenter, S., Karpati, G. and Chang, P. L. (1993). Metachromatic leukodystrophy: multiple nonfunctional and pseudodeficiency alleles in a pedigree: problems with diagnosis and counselling. *Annals of Neurology*. **34**: 212-218.

Fürst, W. and Sandhoff, K. (1992). Activator proteins and topology of lysosomal sphingolipid catabolism. *Biochemica et Biophysica Acta*. **1126**: 1-16.

Garrod, A. E. (1908). Inborn errors in metabolism. Oxford University press. U.K. (reprinted in 1963 with a supplement by H. Harris).

- Gieselmann, V., Polten, A., Kreysing, J. and von Figura, K.** (1989). Arylsulphatase A pseudodeficiency: loss of a polyadenylation signal and N-glycosylation site. *Proceedings of the National Academy of Science*. **86**: 9436-9440.
- Gieselmann, V., Fluharty, A. L., Tonnesen, T. and von Figura, K.** (1991). Mutations in the arylsulphatase A pseudodeficiency allele causing metachromatic leukodystrophy. *American Journal of Human Genetics*. **49**: 407-413.
- Gieselmann, V., Zlotogora, J., Harris, A., Wenger, D. A. and Morris, C. P.** (1994). The molecular genetics of metachromatic leukodystrophy. *Human Mutation*. **4**: 233-242.
- Gieselmann, V.** (1995). Lysosomal storage disorders. *Biochimica et Biophysica Acta*. **1270**: 103-136.
- Goebel, H. H., Busch-Hettwer, H. and Bohl, J.** (1992). Ultrastructural studies of the retina in late-infantile metachromatic leukodystrophy. *Ophthalmic Research*. **24**: 103-109.
- Gravel, R. A., Clarke, J. T. R., Kabeck, M. M., Mahuran, D., Sandhoff, K. and Suzuki, K.** (1995). The G_{M2} Gangliosidoses. In Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D. (eds) *The Metabolic and Molecular Basis of Inherited Disease*. 7th Edition, McGraw-Hill, Inc. Health professionals division.
- Greenfeld, J. G.** (1933). A form of progressive cerebral sclerosis in infants associated with primary degeneration of the interfascicular glia. *Journal of Neurology and Psychopathology*. **13**: 289-302.
- Grunstein, M. and Hogness, D.** (1975). Colony hybridisations: a method for the isolation of cloned DNAs that contain a specific gene. *Proceedings of the National Academy of Science*. **72**: 3961.
- Gustavson, K. H. and Hagberg, B.** (1971). The incidence and genetics of metachromatic leukodystrophy in northern Sweden. *Acta Paediatrica Scandinavica*. **60**: 585-590.

- Hanahan, D.** (1983). Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology*. **166**: 557-580.
- Hasegawa, Y., Kawame, H., Ida, H., Ohashi, E. and Eto, Y.** (1990). Single exon mutation in arylsulphatase A has two effects: loss of enzyme activity and aberrant splicing. *Human Genetics*. **93**: 415-420.
- Heinisch, U., Zlotogora, J., Kafert, S. and Gieselmann, V.** (1995). Multiple mutations are responsible for the high frequency of metachromatic leukodystrophy in a small geographic area. *American Journal of Human Genetics*. **56**: 51-57.
- Henseler, M., Klein, A., Reber, M., Vanier, M. T., Landrieu, P. and Sandhoff, K.** (1996). Analysis of a splice-site mutation in the *sap*-precursor gene of a patient with metachromatic leukodystrophy. *The American Journal of Human Genetics*. **58**: 65-74.
- Hers, H. G.** (1965). Inborn lysosomal disease. *Gastroenterology*. **48**: 625-633.
- Herz, B. and Bach, G.** (1984). Arylsulphatase A in pseudodeficiency. *Human Genetics*. **66**: 147-150.
- Honke, K., Kobayashi, T., Fujii, T., Gasa, S., Xu, M., Takamaru, Y., Kondo, R., Tsuji, S. and Makita, A.** (1993). An adult type metachromatic leukodystrophy caused by substitution of serine for glycine 122 in arylsulphatase A. *Human Genetics*. **92**: 451-456.
- Hopwood, J. J. and Brooks, D. A.** (1996). In *Organelle Disease*, Chapter 6. Ed, Applegarth, Dimmoch and Hall. published by Chapel Hall Medical. In Press.
- Jatzkewitz, H. and Mehl, E.** (1969). Cerebroside-sulphatase and arylsulphatase A deficiency in metachromatic leukodystrophy (ML). *Journal of Neurochemistry*. **16**: 19-28.

- Kappler, J., Pötter, W., Gieselmann, V., Kiessling, W., Friedl, W. and Propping, P.** (1991a). Phenotypic consequence of low arylsulphatase A genotypes (ASA-PD/ASA-PD and ASA-/ASA-PD): does there exist an association with multiple sclerosis ? *Developmental Neuroscience*. **13**: 228-231.
- Kappler, J., Watts, R. W. E., Conzelmann, E., Gibbs, D. A., Propping, P. and Gieselmann, V.** (1991b). Low arylsulphatase A activity and choreoathetotic syndrome in three siblings: differentiation of pseudodeficiency from metachromatic leukodystrophy. *European Journal of Paediatrics*. **150**: 287-290.
- Kappler, J., von Figura, K. and Gieselmann, V.** (1992). Late-onset metachromatic leukodystrophy: molecular pathology in two siblings. *Annals of Neurology*. **31**: 256-261.
- Kappler, J., Sommerlade, H. J., von Figura, K. and Gieselmann, V.** (1994). Complex arylsulphatase A alleles causing metachromatic leukodystrophy. *Human Mutation*. **4**: 119-127.
- Kawaguchi, Y., Okamoto, T., Taniwaki, M., Aizawa, M., Inoue, M., Katayama, S., Kawakami, H., Nakamura, S., Nishimura, M., Akiguchi, I.,** (1994). CAG expansions in a novel gene for Machado-Joseph disease at chromosome 14q32.1. *Nature Genetics*. **8**: 221-228.
- Kolodny, E. H. and Fluharty, A. L.** (1995). Metachromatic leukodystrophy and multiple sulphatase deficiency: sulphatide lipidosis. In Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D. (eds) *The Metabolic and Molecular Basis of Inherited Disease*. 7th Edition, .McGraw-Hill, Inc. Health professionals division.
- Kornfeld, S.** (1992). Structure and function of the mannose 6-phosphate/insulinlike growth factor II receptors. *Annual Review of Biochemistry*. **61**: 307-330.

- Kornfeld, R. and Kornfeld, S.** (1985). Assembly of asparagine-linked oligosaccharides. *Annual Review of Biochemistry*. **54**: 631-634.
- Kreysing, J., Bohne, W., Bösenberg, C., Marchesini, S., Turpin, J. C., Baumann, N., von Figura, K. and Gieselmann, V.** (1993). High residual arylsulphatase A activity in a patient with late-infantile metachromatic leukodystrophy. *American Journal of Human Genetics*. **53**: 339-346
- Kiesewetter, S., Macek, M., Davis, C., Curristin, S. M., Chu, C.-S., Graham, C., Shrimpton, A. E., Cashman, S. M., Tsui, L.-C., Mickle, J., Amos, J., Highsmith, W. E., Shuber, A., Witt, D. R., Crystal, R. G. and Cutting, G. R.** (1993). A mutation in CFTR produces different phenotypes depending on chromosomal background. *Nature Genetics*. **5**: 274-278.
- Kihara, H., Ho, C.-K., Fluharty, A. L., Tsay, K. K. and Hartlage, P. L.** (1980). Prenatal diagnosis of metachromatic leukodystrophy in a family with pseudo arylsulphatase A deficiency by the cerebroside sulphate loading test. *Paediatric Research*. **14**: 224-227.
- Klein, A., Henseler, M., Klein, C., Suzuki, K., Harzer, K. and Sandhoff, K.** (1994). Sphingolipid activator protein D (*sap-D*) stimulates the lysosomal degradation of ceramide *in vivo*. *Biochemical and Biophysical Research Communications*. **200**: 1440-1448.
- Kondo, R., Wakamatsu, N., Yoshino, H., Fukuhara, N., Miyatake, T. and Tsuji, S.** (1991). Identification of a mutation in the arylsulphatase A gene of a patient with adult-type metachromatic leukodystrophy. *American Journal of Human Genetics*. **48**: 971-978.
- Kreysing, J., von Figura, K. and Gieselmann, V.** (1991). Structure of the arylsulphatase A gene. *European Journal of Biochemistry*. **191**: 627-631.

Kreysing, J., Bohne, W., Bosenberg, C., Marchesini, S., Turpin, J. C., Baumann, N., von Figura, K. and Gieselmann, V. (1993). High residual arylsulphatase A (ARSA) activity in a patient with late-infantile metachromatic leukodystrophy. *American Journal of Human Genetics*. **53**: 339-346.

Krivit, W., Sharpiro, E., Kennedy, W., Lipton, M., Lockman, L., Smith, S., Summers, C. G., Wenger, D. A., Tsai, M. Y., Ramsay, N. K. C., Kersey, J. H., Yao, J. K. and Kaye, E. (1990). Treatment of late-infantile metachromatic leukodystrophy by bone marrow transplantation. *New England Journal of Medicine*. **322**: 28-32.

Krivitt, W., Sharpiro, E., Lockman, L., Kennedy, W., Dhuna, A., Ringden, O., Henslee-Downey, J., Yeager, A., Wenger, D. and Bayever, E. (1992). Recommendations for treatment of metachromatic leukodystrophy by bone marrow transplantation based on a review of seven patients who have been engrafted for at least one year. In *Correction of Certain Genetic Diseases by Transplantation*. London Westminster Medical School Press, Ed Hobbs, J. R.

Lagnado, C. A., Brown, C. Y. and Goodall, G. J. (1994). AUUUA is not sufficient to promote poly(A) shortening and degradation of an mRNA: the functional sequence within the AU-rich elements may be UUAUUUA(U/A)(U/A). *Molecular and Cellular Biology*. **14**: 7984-7995.

Langdown, J. V., Williamson, D., Knight, C. B., Rubenstein, D. and Carrell, R. W. (1989). A new doubly substituted sickling haemoglobin: HbS-Oman. *British Journal of Haematology*. **71**:443-444.

Langenbeck, V., Dunker, P., Heipertz, R. and Pilz, H. (1977). Inheritance of metachromatic leukodystrophy. *American Journal of Human Genetics*. **29**: 638-639.

- Leabeck, D. H. and Walker, P. G.** (1961). Studies on glucosaminidase. The fluorimetric assay of N-acetyl-B-glucosaminidase. *Biochemistry Journal*. **78**: 151-156.
- Lee-Vaupel, M. and Conzelmann, E.** (1987). A simple chromogenic assay for arylsulphatase A. *Clinica. Chimica. Acta*. **164**: 171-180.
- Leinekugel, P., Michel, S., Conzelmann, E. and Sandhoff, K.** (1992). Quantitative correlation between the residual activity of B-hexosaminidase and arylsulphatase A and the severity of the resulting lysosomal storage disease. *Human Genetics*. **88**: 513-523.
- Leistner, S., Young, E., Meaney, C. and Winchester, B.** (1995). Pseudodeficiency of arylsulphatase A: strategy for clarification of genotype in families of subjects with low ASA activity and neurological symptoms. *Journal of Inherited Metabolic Disease*. **18**: 710-716.
- Li, Y.-T. and Li, S.-C.** (1984). Activator proteins related to the hydrolysis of glycosphingolipids catalysed by lysosomal glycosidases. In *Lysosomes in Biology and Pathology*. Elsevier Science Publishers B. V., edited by Dingle, J. T., Dean, R. T. and Sly, W.
- Lott, I. T., Dulaney, J. T., Milunsky, A., Hoefnagel, D. and Moser, H. W.** (1976). Apparent biochemical homozygosity in two obligate heterozygotes for metachromatic leukodystrophy. *Journal of Paediatrics*. **89**: 438-440.
- Manowitz, P., Kling, A. and Kohn, H.** (1978). Clinical course of adult metachromatic leukodystrophy presenting as schizophrenia. *Journal of Nervous and Mental Disease*. **166**: 500-506.
- Matovcik, L. M., Goodhouse, J. and Farquhar, M. G.** (1990). The recycling itinerary of the 46 kDa mannose 6-phosphate receptor - Golgi to late-endosomes - coincides with that of the 215 kDa M6PR. *European Journal of Cell Biology*. **96**: 644-650.
- Mehl, E. and Jatzkewitz, H.** (1965). Evidence for the genetic block in metachromatic leukodystrophy. *Biochemical and Biophysical Research Communications*. **19**: 407-411.

- MacFaul, R., Cavanagh, N., Lake, B. D., Stephens, R. and Whitfield, A. E.** (1982). Metachromatic leukodystrophy: a review of 38 cases. *Archives of Disease in Childhood*. **57**: 168-175.
- McKusick, V. A., Howell, R. R., Hussels, I. E., Neufeld, E. F. and Stevenson, R. E.** (1972). Allelism, non-allelism and genetic compounds among the mucopolysaccharidoses. *The Lancet*. **1**: 993-996.
- McKusick, V. A.** (1973) Phenotypic diversity of human diseases resulting from allelic series. *American Journal of Human Genetics*. **25**: 446-456.
- McKusick, V. A.** (1994). *Mendelian inheritance in man: a catalogue of human genes and genetic disorders*. 11th edition. Johns Hopkins University Press.
- Moser, H. W., Moser, A. B. and McKhann, G. M.** (1967). The dynamics of lipidosis: turnover of sulphatide, steroid sulphate and polysaccharide sulphate in metachromatic leukodystrophy. *Archives of Neurology*. **17**: 494-511.
- Moser, H. W., Smith, K. D. and Moser, A. B.** (1995). X-linked adrenoleukodystrophy. In *The Metabolic and Molecular Basis of Inherited Disease*. 7th Edition, .McGraw-Hill, Inc. Health professionals division. Edited by Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D.
- Murphy, H. R., Kalman, M. and Cashel, M.** (1992). Identification of the *gppB* locus as two convergent arylsulphatase-like genes, *aslA* and *aslB*, capable of suppressing a guanosine pentaphosphate phosphatase missense mutation in *Escherichia coli*. (Unpublished GenBank entry ECOASLAB).
- Murray, V.** (1989). Improved double-stranded DNA sequencing using the linear polymerase chain reaction. *Nucleic Acids Research*. **17**: 8889.

- Myerowitz, R. and Costigan, F. C.** (1988). The major defect in Ashkenazi Jews with Tay-Sachs disease is an insertion in the gene for the alpha-chain of b-hexosaminidase. *Journal of Biological Chemistry*. **263**: 18587-18589.
- Navon, R. and Proia, R. L.** (1989). The mutations in Ashkenazi Jews with adult G_{M2} gangliosidosis, the adult form of Tay-Sachs disease. *Science*. **243**: 1471-1474.
- Nelson, P. V., Carey W. F. and Morris, C. P.** (1991). Population frequency of the arylsulphatase A pseudo-deficiency allele. *Human Genetics*. **87**: 87-88.
- Neufeld, E. F. and Muenzer, J.** (1989). The mucopolysaccharidoses. In Scriver, C.R., Beaudet, A.L., Sly, W.S., and Valle, D. (eds): *The Metabolic Basis of Inherited Disease*. 6th edn., McGraw-Hill, New York. pp. 1565-1587.
- Neufeld, E. F.** (1991). Lysosomal storage diseases. *Annual Review of Biochemistry*. **60**: 257-280.
- Norton, W. T. and Poduslo, S. E.** (1973). Myelination in rat brain: changes in myelin composition during brain maturation. *Journal of Neurochemistry*. **21**: 759-773.
- O'Brien, J. S. and Kishimoto, Y.** (1991). Saposin proteins: structure, function, and role in human lysosomal storage disorders. *The FASEB Journal*. **5**: 301-308.
- Orita, M., Youichi, S., Sekiya, T. and Hayashi, K.** (1989). Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics*. **5**: 874-879.
- Orr, H. T., Chung, M. Y., Banfi, S., Kwiatkowski, T. J., Servadio, A., Beaudet, A. L., McCall, A. E., Duvick, L. A., Ranum, L. P. and Zoghbi, H. Y.** (1993). Expansion of an unstable trinucleotide repeat in spinocerebellar ataxia type I. *Nature Genetics*. **4**: 221-226.
- Pastor-Soler, N. M., Rafi, M. A., Hoffman, J. D., Hu, D. and Wenger, D. A.** (1993). Metachromatic leukodystrophy among the Navajo indians: a study of arylsulphatase A

mutations present in this population. *American Journal of Human Genetics*. **53**: 940 (abstract).

Paul, J. (1972). *Cell and tissue culture*. Fourth Edition. Churchill Livingstone, Edinburgh and London.

Pfeffer, S. R. (1988). Mannose 6-phosphate receptors and their role in targeting proteins to the lysosomes. *Journal of Membrane Biology*. **103**: 7-16.

Penzein, J. M., Kappler, J., Herschkowitz, N., Schuknecht, B., Leinekugel, P., Propping, P., Sangiorgi, S., Ferlini, A., Zanetti, A. and Mochi, M. (1991). reduced activity of arylsulphatase A and predisposition to neurological disorders. *American Journal of Medical Genetics*. **40**: 365-369.

Penzein, J. M., Kappler, J., Herschkowitz, N., Schknecht, B., Leinekugel, P., Propping, P., Tønnesen, T., Lou, H., Moser, H., Zierz, S., Conzelmann, E. and Gieselmann, V. (1993). Compound heterozygosity for metachromatic leukodystrophy and arylsulphatase A alleles is not associated with progression of neurological disease. *American Journal of Human Genetics*. **52**: 557-564.

Peters C, Schmidt B, Rommerskirch W, Rupp K, Zuehlendorf M, Vingron M, Meyer HE, Pohlmann R, von Figura K (1990) Phylogenetic conservation of arylsulphatases: cDNA cloning and expression of human arylsulphatase B. *Journal of Biological Chemistry*. **265**: 3374-3381.

Philippart, M., Sarlieve, L., Meurant, C. and Mechler, L. (1972). Human urinary cerebroside sulphates in patients with sulfatidosis (metachromatic leukodystrophy). *Journal of Lipid Research*. **12**: 434.

- Polten, A., Fluharty, A. L., Fluharty, C. B., Kappler, J., von Figura, K. and Gieselmann, V.** (1991). Molecular basis of different forms of metachromatic leukodystrophy. *New England Journal of Medicine*. **324**: 18-22.
- Porter, M. T., Fluharty, A. L., De-la-Flor, S. D. and Kihara, H.** (1972). Cerebroside sulfatase determination in cultured human fibroblasts. *Biochimica Biophysica Acta*. **258**: 769-778.
- Poulos, A. and Beckman, K.** (1981). The effect of the interdependence of protein and bile salt concentrations on the measurement cerebroside sulphate sulphatase activity in human leukocyte and fibroblast extracts. *Biochimica Biophysica Acta*. **615**: 370-380.
- Ranum, L. P., Lundgren, J. K., Schut, L. J., Ahrens, M. J., Perlman, S., Aita, J., Bird, T. J., Gomez, C. and Orr, H. T.** (1995). Spinocerebellar ataxia type I and Machado-Joseph disease: incidence of CAG expansions among adult-onset ataxia patients from 311 families with dominant, recessive, or sporadic ataxia. *American Journal of Human Genetic*. **57**: 603-608.
- Reed, K. C. and Mann, D. A.** (1985). Rapid transfer of DNA from agarose gels to nylon membranes. *Nucleic Acids Research*. **13**: 7207-7221.
- Reider-Grosswasser, I. and Bornstein, N** (1987). CT and MRI in late-onset metachromatic leukodystrophy. *Acta Neurology Scandinavia*. **75**: 64-69.
- Robertson, D. A., Freeman, C., Morris, C. P., Hopwood, J. J.** (1992). A cDNA clone for glucosamine-6-sulphatase reveals differences between aryl- and non-arylsulphatases. *Biochemistry Journal*. **288**: 539-544.
- Romeo, G. and McKusick, V. A.** (1994). Phenotypic diversity, allelic series and modifier genes. *Nature Genetics*. **7**: 451-453.

- Rootwelt, H., Brodtkorb, E. and Kvittingen, E. A.** (1994). Identification of a frequent pseudodeficiency mutation in the fumarylacetoacetase gene, with implications for diagnosis of tyrosinemia type I. *American Journal of Human Genetics*. **55**: 1122-1127.
- Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A.** (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**: 487-491.
- Sambrook, J., Fritsch, E. F. and Maniatis, T.** (1989). Molecular Cloning: a laboratory manual. Second Edition. Cold Spring Harbor Press.
- Sandhoff, K., Harzer, K. and Furst, W.** (1995). Sphingolipid activator proteins. In *The Metabolic and Molecular Basis of Inherited Disease*. 7th Edition, McGraw-Hill, Inc. Health professionals division. Edited by Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D.
- Sangiorgi, S., Ferlini, A., Zanetti, A. and Mochi, M.** (1991). Reduced activity of arylsulphatase A and predisposition to neurological disorders: analysis of 140 pediatric patients. *American Journal of Medical Genetics*. **40**: 365-369.
- Sasaki H, Yamada K, Akasaka H, Suzuki K, Saito A, Sato M, Shimada H** (1988) cDNA cloning, nucleotide sequence and expression of the gene for arylsulphatase in the sea urchin (*Hemicentrotus pulcherrimus*) embryo. *European Journal of Biochemistry*. **177**: 9-13.
- Sawadogo, M. and Van Dyke, M. W.** (1991). A rapid method for the purification of deprotected oligodeoxynucleotides. *Nucleic Acids Research*. **19**: 674.
- Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D.** (1995). The metabolic and molecular bases of inherited disease. Seventh Edition. McGraw-Hill, Inc. Health professionals division.

- Shapiro, M. B and Senapathy, P.** (1987). RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. *Nucleic Acids Research*. **15**: 7155-7174.
- Sharpiro, E. G., Lockman, L. A., Knopman, D. and Krivit, W.** (1994). Characteristics of dementia in late-onset metachromatic leukodystrophy. *Neurology*. **44**: 662-665.
- Shen, N., Li, Z.-G., Waye, J. S., Francis, G. and Chang, P. L.** (1993). Complications in the genotypic molecular diagnosis of pseudo arylsulphatase A deficiency. *American Journal of Medical Genetics*. **45**: 631-637.
- Siegel, E. G., Lücke, H., Schauer, W. and Creutzfeldt, W.** (1992). Repeated upper gastrointestinal haemorrhage caused by metachromatic leukodystrophy of the gall bladder. *Digestion*. **51**: 121-124.
- Simpson, N. E. and Kalow, W.** (1963). Serum cholinesterase levels in families and twins. *American Journal of Human Genetics*. **15**: 280-287.
- Stein, C., Gieselmann, V., Kreysing, J., Schmidt, B., Pohlmann, R., Waheed, A., Meyer, H. E., O'Brien, J. S. and von Figura, K.** (1989). Cloning and expression of human arylsulphatase A. *Journal of Biochemistry*. **264**: 1252-1259.
- Taroni, F., Verderio, E., Fiorucci, S., Cavadini, P., Finocchiaro, G., Uziel, G., Lamantea, E., Gellera, C. and DiDonato, S.** (1992). Molecular characterisation of inherited caraitine palmitoyltransferase II deficiency. *Proceedings of the National Academy of Science*. **89**: 8429-8433.
- Thomas, P. K., King, R. H. M., Kocen, R. S. and Brett, E. M.** (1977). Comparative ultrastructural observations on peripheral nerve abnormalities in the late infantile, juvenile and late onset forms of metachromatic leukodystrophy. *Acta Neuropathology (Berlin)*. **39**: 237-245.

- Thomas, G. H.** (1994). "Pseudodeficiencies" of lysosomal hydrolyases. *American Journal of Human Genetics*. **54**: 934-940.
- Thuring, R. W. J., Sanders, J. P. M. and Borst, P.** (1975). A freeze-squeeze method for recovering long DNA from agarose gels. *Analytical Biochemistry*. **66**: 213-220.
- Tinuper, P., Plazzi, G., Monari, L., Sangiorgi, S., Pellissier, J.-F., Cerulla, A., Provini, F., Capellari, S., Baruzzi, A., Lugaresi, E. and Montagna, P.** (1994). Arylsulphatase A pseudodeficiency and lafora bodies in a patient with progressive myoclonic epilepsy. *Epilepsia*. **35**: 332-335.
- Tomatsu, S., Fukuda, S., Masue, M., Sukegawa, K., Fukao, T., Yamagishi, A., Hori, T., Iwata, H., Ogawa, T., Nakashima, Y., Hanyu, Y., Hashimoto, T., Titani K., Oyama, R., Suzuki, M., Yagi, K., Hayashi, Y. and Orii, T.** (1991). Morquio disease: isolation, characterization and expression of full-length cDNA for human N-acetylgalactosamine-6-sulphate sulphatase. *Biochemica Biophysica Research Communications*. **181**: 677-683.
- Tomczak, J., Boogen, C. and Grebner, E. E.** (1993). Distribution of a pseudodeficiency allele among Tay-Sachs carriers. *The American Journal of Human Genetics*. **53**: 537-539.
- Tonnesen, T., Lou, H., Moser, H., Zierz, S., Conzelmann, E. and Gieselmann, V.** (1993). Compound heterozygosity for metachromatic leukodystrophy and arylsulphatase A pseudodeficiency alleles is not associated with progressive neurological disease. *American Journal of Human Genetics*. **52**: 557-564.
- Triggs-Raine, B. L., Mules, E. H., Kaback, M. M., Lim-Steele, J. S. T., Dowling, C. E., Akerman, B. R., Natowicz, M. R., Grebner, E. E., Navon, R., Welch, J. P., Greenberg, C. R., Thomas G. H. and Gravel, R. A.** (1992). A pseudodeficiency allele common in non-Jewish Tay-Sachs carriers: implications for carrier screening. *American Journal of Human Genetics*. **51**: 793-801.

- Tso, J. Y., Sun, X.-H., Kao, T.-H., Davis, K. S. and Wu, R. (1985).** Isolation and characterisation of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: genomic complexity and molecular evolution of the gene. *Nucleic Acids Research*. **13**: 2485-2502.
- Vervoort, R., Islam, M. R., Sly, W., Chabas, A., Wevers, R., de Jong, J., Liebaers, I. and Lissens, W. (1995).** A pseudodeficiency allele (D152N) of the human β -glucuronidase gene. *American Journal of Human Genetics*. **57**: 798-804.
- von Figura, K. and Hasilik, A. (1986).** Lysosomal enzymes and their receptors. *Annual Review of Biochemistry*. **55**: 167-193.
- Woollart, E., Harvey, J. S., Callen, D. F., Carey, W. F. and Morris, C. P. (1996).** Arylsulphatase A lysosomal enzyme map position 22q13.3. *Chromosome Research*. **4**: 407.
- Warfel, K. A. and Hull, M. T. (1984).** Villous papilloma of the gallbladder in association with leukodystrophy. *Human Pathology*. **15**: 1192-1194.
- Warner, J. O. (1975).** Juvenile onset metachromatic leukodystrophy: failure of response on a low vitamin A diet. *Archives of Diseases of Childhood*. **50**: 735-737.
- Weinberger, L. M., Schmidley, J. W., Schafer, I. A. and Raghaven, S. (1994).** Delayed postanoxic demyelination and arylsulphatase A pseudodeficiency. *Neurology*. **44**: 152-154.
- Wenger, D. A. and Louie, E. (1991).** Pseudodeficiencies of arylsulphatase A and galactocerebrosidase activities. *Developmental Neuroscience*. **13**: 216-22.
- Wiesmann, U. N., Rossi, E. E. and Herschkowitz, N. N. (1971).** Treatment of metachromatic leukodystrophy in fibroblasts by enzyme replacement. *New England Journal of Medicine*. **284**: 672-673.

- Wilson, P. J., Morris, C. P., Anson, D. S., Occhiodoro, T., Bielicki, J., Clements, P. R., Hopwood, J. J.** (1990). Hunter syndrome: isolation of an iduronate-2-sulphatase cDNA clone. *Proceedings of the National Academy of Science USA*. **87**: 8531-8535.
- Yang, Q., Angerer, L. M. and Angerer, R. C.** (1989) Structure and tissue-specific developmental expression of a sea urchin arylsulphatase gene. *Developmental Biology*. **135**: 53-65.
- Yanisch-Perron, C., Vieira, J. and Messing, J.** (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene*. **33**: 103-119.
- Yen, P. H., Allen, E., Marsh, B., Mohandas, T., Wang, N., Taggart, R. T. and Shapiro, L. J.** (1987). Cloning and expression of steroid sulphatase cDNA and the frequent occurrence of deletions in STS deficiency: Implications for X-Y interchange. *Cell*. **49**: 443-454.
- Young, E., Willcox, P., Whitfield, A. E. and Patrick, A. D.** (1975). Variability of acid hydrolase activities in cultured skin fibroblasts and amniotic fluid cells. *Advances in Experimental and Medical Biology*. **53**: 155-169.
- Zlotogora, J., Costeff, H. and Elian, E.** (1981). Early motor development in metachromatic leukodystrophy. *Archives of Disease of Children*. **59**: 309-310.
- Zlotogora, J., Furman-Shaharabani, Y., Goldenfum, S., Winchester, B., von Figura, K. and Gieselmann, V.** (1994). Arylsulfatase A pseudodeficiency: a common polymorphism which is associated with a unique haplotype. *American Journal of Medical Genetics*. **52**: 146-150.
- Zlotogora, J. Bach, G., Bösenberg, C., Barak, Y., von Figura, K. and Gieselmann, V.** (1995). Molecular basis of late-infantile metachromatic leukodystrophy in the Habbanite Jews. *Human Mutation*. **5**: 137-143.

