THE MOLECULAR GENETICS OF FUCOSIDOSIS.

This thesis is submitted in fulfilment of the conditions governing candidates for the degree of

DOCTOR OF PHILOSOPHY

in the

FACULTY OF LIFE SCIENCES

of the

UNIVERSITY OF LONDON

by

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May 1992

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Abstract.

Fucosidosis is a rare, autosomal recessive lysosomal storage disease resulting from a deficiency of α–L-fucosidase. The molecular basis of fucosidosis was investigated in six unrelated patients. The residual α-L-fucosidase activity in extracts of fibroblasts, leukocytes and plasma has been characterised and the cross reacting immunological material (CRIM) against human α-L-fucosidase determined using a two antibody sandwich ELISA technique. Four of the patients had negligible α-L-fucosidase activity and very low CRIM levels, which is typical of fucosidosis patients. Typical patterns of storage products were also seen in urine of these patients by thin layer chromatography (TLC). One atypical patient had intermediate activity and intermediate CRIM. The α-L-fucosidase activity in the sixth patient increased from low levels at age two years to control levels at ten years of age. CRIM levels were above that of controls. This atypical patient also had a different pattern of storage products in urine from that typically seen in fucosidosis or control urine.

Analysis of DNA extracted from fibroblasts or lymphocytes revealed that none of the six patients have either of the two previously described fucosidosis-causing mutations. The DNA was haplotyped by two previously described RFLP's observed with Pvu11 and Bgl1 restriction enzymes. Southern blotting also showed that none of the patients had any gross gene alterations such as large insertions, deletions or rearrangements. The products of amplification by PCR of exon 8 and flanking regions from all six patients were analysed by mismatch analysis and direct sequencing. No mutations were found in this region.

A mutation in the highly conserved donor splice site sequence of intron 5 was identified in one Asian patient. The G-> A transition at the first nucleotide of intron 5 presumably results in aberrant mRNA splicing. The mutation generates a new Taq1 site and cosegregates with the mutant allele in the family of the patient, as determined by enzyme activity.

Canine fucosidosis can be used as a model for the human disease. The α–L-fucosidase activity, storage products and mRNA expression was characterised in two fucosidosis dogs. DNA from affected dogs and controls was used by Southern blotting with

human cDNA fucosidase probes with the aim of detecting RFLPs. A restriction map of the canine gene was constructed and one RFLP was found with Xba1 , which did not segregate with the disease.

Mannose-binding protein is a mammalian lectin with a role in host defence. A human liver cDNA library was screened with rat MBP-C cDNA with the aim of isolating the human MBP sequence.

Acknowledgements.

I would especially like to thank Dr Bryan Winchester for his kindness and patience in the supervision of this work.

I would also like to thank my colleagues in the Clinical Biochemistry Department, especially Elizabeth Young, Tony Whitfield, Stella Geddes, Dr.Samer El Daher and Helen Cragg; and all those in the Genetics department, especially Paul Rutland and Sandra Strautnieks, for their help and advice. I would also like to express my gratitude to Francis Shanahan for her help at the beginning of the project and to Dr. P. Willems for my interesting and stimulating visit to his laboratory in Antwerp.

Finally I would like to acknowledge the generous contributions of cDNA probes from Dr.J.S. O'Brien and Dr. K. Drickamer; antibodies from Dr. Alhadeff and sequence information from Dr.K.Kretz.

Erratum.

The names of restriction enzymes should be as follows:

*Alu*I, *Ava*II, *Bam*HI, *Bcl*I, *Bgl*I, *Bgl*II, *Bst*EII, *Eco*RI, *Eco*RV, *Hin*cII, *Hin*dIII, *Hin*fI, *Kpn*I, *Mbo*I, *Msp*I, *Pst*I, *Pvu*I, *Pvu*II, *Rsa*I, *Sac*I, *Ssp*I, *Stu*I, *Taq*I, *Xba*I, *Xmn*I.

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CHAPTER 1

Introduction.

1.1. Lysosomes and lysosomal storage diseases.

1.1a. Lysosomes.

The presence of a membrane-bound organelle containing hydrolytic enzymes was first implicated by the observation of latency in acid phosphatase activity, due to a membrane-like barrier limiting the access of enzyme to substrate (de Duve et al., 1955). Lysosomes were subsequently visualised by Novikoff (reviewed by Bainton, 1981).

Lysosomes have a role in the breakdown of endogenous or internalised macromolecules following autophagy or endocytosis. The smaller products of catabolism either diffuse out of the lysosome into the cytoplasm, or are carried out by specific transport mechanisms. Lysosomes also have a role in the processing of proteins, proteolytic activation of hormones and regulation of cholesterol synthesis. The internal low pH of lysosomes is maintained by a proton pump in the lysosomal membrane. Lysosomes provide an enclosed acidic environment where hydrolytic enzymes that are harmful to the rest of the cell are segregated.

1.1b. Lysosomal enzymes.

Lysosomes contain at least 60 different enzymes with acidic pH-optima, which catalyse breakdown of polypeptides, the carbohydrate side chains of glycoproteins and glycolipids, lipids, esters, glycosaminoglycans and nucleic acids. Catabolism is carried out in an ordered stepwise manner, each enzyme having a high substrate specificity.

Initially secretory, membrane and lysosomal proteins share a common pathway of biosynthesis and processing (reviewed by Kornfeld, 1986). Synthesis occurs on membrane-bound polysomes of the rough endoplasmic reticulum (RER), and a hydrophobic signal sequence allows translocation of the nascent polypeptide into the lumen of the RER. The polypeptides are cotranslationally glycosylated at selected asparagine residues. The signal

sequence is cleaved and initial processing of the asparagine-linked oligosaccharides commences. The glycosylated polypeptides are translocated from the ER to the Golgi apparatus by vesicular transport. At this point, the three classes of proteins undergo a variety of post-translational modifications to enable segregation and targetting to their final destination. For lysosomal enzymes this involves addition of a phosphate group to specific mannose residue(s) on the oligosaccharide chain of the glycoprotein. Phosphorylated high mannose-type oligosaccharides are heterogenous with respect to the number of phosphorylated mannose residues and their position within the oligosaccharide. N acetyl glucosaminyl phosphotransferase catalyses transfer of N acetyl glucosamine 1 phosphate from UDP N acetyl glucosamine to C6 of a specific mannose residue. Phosphorylation occurs in two sequential steps: a phosphate group is attached to mannose residue(s) on the 1,6 branch of high mannose oligosaccharides in the pre Golgi and to the 1,3 branch in the Golgi. The two step phosphorylation occurs by a common phosphotransferase and results from a change in the conformation of the oligosaccharide through the action of Golgi associated α-mannosidase 1 (Lazarino and Gabel, 1989). N acetyl glucosamine phosphodiesterase then releases N acetyl glucosamine thereby exposing the phosphate group. The phosphorylating enzyme probably recognises a number of topological features on the surface of the polypeptide shared by lysosomal enzymes (Baranski et al., 1990). The phosphomannosyl residue serves as a high affinity ligand for binding of mannose-6-phosphate receptors (MPR) in the Golgi. The ligand receptor complex exits the Golgi via coated vesicles and enters a prelysosomal compartment where dissociation occurs due to the low pH. The receptor returns to the Golgi or is moved to the plasma membrane where exogenous lysosomal enzymes are bound and internalised. Two mannose 6 phosphate receptors have been identified, a cation-independent one of Mr 215,000 (CIMPR) and a cation-dependent one of Mr 46,000 (CDMPR). Both receptors have been cloned and their structures characterised (Oshima et al., 1988, Pohlman et al., 1987). Both function in lysosomal enzyme sorting and cycle to the plasma membrane. However only the CIMPR is capable of binding and internalising lysosomal enzymes at the plasma membrane. The CIMPR is identical to the insulin-like growth factor 11 receptor (Morgan et al., 1987). It is unknown why two receptors are present in the cell. (For a review see Dahms, 1989). The mannose-6-phosphate pathway is not involved in the targetting

of lysosomal acid phosphatase (LAP) to lysosomes. LAP is synthesised as a transmembrane protein and is transported to the lysosomes via the cell surface. It is subsequently cleaved by a cytoplasmic thiol protease and a lysosomal aspartyl protease. Internalisation is dependent on the presence of a tyrosine residue in the cytoplasmic tail (Peters et al., 1990). Glucocerebrosidase also does not acquire phosphorylated mannose residues (Aerts et al., 1988) for lysosomal targetting. Efficient targetting of lysosomal membrane glycoproteins to lysosomes appears to require a tyrosine residue at a specific position in the cytoplasmic domain of the glycosylated protein (Williams and Fukuda, 1990).

The genes for several of the lysosomal enzymes have been cloned (Table 1.1).

1.1c. Alternative transcripts of lysosomal enzymes.

Multiple transcripts have been identified from a number of genes encoding lysosomal enzymes including β-glucuronidase, sphingomyelinase, β-galactosidase, β-Nacetyl galactosaminidase and N-aspartyl-β glucosaminidase as well as the sulfatide activator gene, probably resulting from alternative splicing (Oshima et al., 1987; Schuchman et al., 1991; Morreau et al., 1989; Yamauchi et al., 1990; Fischer and Aronson., 1991; Holtschmidt et al., 1991). In the cases of sphingomyelinase, β-galactosidase and β-glucuronidase only the full length transcript produces a catalytically active enzyme.

1.1d. Activator proteins and protection factors.

Certain lysosomal enzymes require activating or protection factors for activity. These include spingolipid activator proteins (saposins), the 32kDa protective factor for β-galactosidase and sialidase which is essential for the stability in vivo of both enzymes (D'Azzo et al., 1982), and the activator protein of N-acetylhexosaminidase A which is necessary for the interaction of a lipid substrate and water-soluble enzyme.

Saposins A, B, C and D are proteolytically cleaved from a single precursor protein encoded by the prosaposin gene. The prosaposin cDNA has been cloned and sequenced and

Enzyme.	Disease.	Gene size.	Chromosome location.	
1. Mucopolysaccharidosis.				
alpha-L-iduronidase.	Hurler	8kb, 12 exons.	4p16.3	Scott; 1990,1991
alpha-L-iduronidase sulphate sulphatase	Hunter.		Xq28	Wilson;1990
beta-D-glucuronidase	Sly (MPS V11)	~21kb, 12exons	7q21-22	Oshima; 1987
Arylsulphatase B	Maroteaux Lamy (MPS V1)		5q13-14	Schuchman;1990
2. Glycoprotein metabolism.				
alpha-L-fucosidase	Fucosidosis.	~22kb, 8 exons	1p32-34	Fukushima;1985
Glycosylasparaginase.	Aspartylglucosaminuria.		4q	Ikonen;1991
3. Sphingolipidosis and neutral lipidosis.				
alpha-D-galactosidase	Fabry.	12kb, 7 exons.	Xq21.33-22	Kornreich;1989
beta-D-glucocerebrosidase	Gaucher.	7.6kb, 11 exons.	1q21	Sorge;1985. Horowitz;1989
beta-hexosaminidase, alpha subunit	Tay Sachs.	35-40kb, 14 exons	15q23-24	Myerowitz;1985
beta-hexosaminidase, beta subunit	Sandhoff.	45kb, 14 exons.	5q11	O'Dowd ;1985
Sphingomyelinase	Nieman Pick A and B..	4.7kb, 6 exons	11p15.1-15.4	Quintern; 1989. da-Veiga-Pereira;1991
Arylsulphatase A	Metachromatic Leukodystrophy.	3.2kb, 8 exons.	22q13	Stein; 1989. Kreysing;1990
alpha-glucosidase	Pompe.	20kb, 20 exons.	17q21-23	Hoefsloot; 1988,1990.
beta-galactosidase	GM1 gangliosidosis, Morquio B (MPS1VB)		3	Oshima;1988.
alpha-N-acetylgalactosaminidase	Schlinder.	13.7kb, 9 exons.	22q13-qter	Wang; 1991
Prosaposin (Saposin A,B,C,D)	Activator deficient Metachromatic Leukodystrophy.		10q21-22	O'Brien; 1988

Table 1.1. Cloned lysosomal enzyme genes.

codes for a 524 amino acid glycoprotein with four domains of approximately 80 amino acids (O'Brien et al.,1988). Saposins A and C specifically activate hydrolysis of glucocerebroside by glucosylceramidase and galactocerebroside by galactosylceramidase (Morimoto et al., 1989). Saposin D activates hydrolysis of sphingomyelin by sphingomyelin phosphodiesterase (Morimoto et al., 1988). Saposin B stimulates hydrolysis of galactocerebroside sulphate by aryl sulphatase A, GM1 ganglioside by acid galactosidase and globotriaosyl ceramide by galactosidase A (Li et al., 1985). Saposins A, C, and D, appear to act by binding to the respective enzymes and increasing the maximal velocity of hydrolysis and lowering the Km. Conversly saposin B interacts with lipid substrates solubilising them for enzymatic hydrolysis.

1.1e. Lysosomal storage diseases.

The majority of lysosomal storage diseases result from a defect in the gene encoding a lysosomal enzyme causing deficient activity of that enzyme. Some lysosomal storage diseases however result from mutations in genes other than those encoding lysosomal hydrolases. Cystinosis, a form of Vitamin B12 deficiency and sialic acid storage disease result from a defect in transport across the lysosomal membrane of cystine, Vitamin B12 and sialic acid respectively . Mucolipidosis 11 and 111 result from a deficiency of the enzyme responsible for the addition of a phosphate group to mannose residues of lysosomal enzymes resulting in erroneous targetting of the lysosomal enzymes. A fourth group of lysosomal storage diseases, such as galactosialidosis, is due to a deficiency of activating proteins or protective factors. The deficient activity of a lysosomal enzyme results in the intralysosomal accumulation of the intermediates of catabolism. Accumulation of storage products in the lysosomes is the initial step in the process that leads to the clinical symptoms of lysosomal storage diseases (Table 1.2). The influx of a particular substrate can vary between tissue type and developmental stage and will determine which tissues are more severely affected.

All the lysosomal storage disorders so far identified have been autosomal recessive, with the exceptions of Hunter and Fabry diseases, which are X-linked.

Lysosomal storage diseases.

Disorder	Defect	McKusick No.
1.Deficiency of lysosomal enzymes.		
Sphingolipidoses and neutral lipidoses.		
Fabry	α -D-galactosidase A	30150
Farber	Ceramidase	22800
Gaucher	Glucocerebrosidase	23080
GM1 Gangliosidosis	β-D-galactosidase	23050
GM2 Gangliosidosis:		
Tay Sachs	β–Hexosaminidase A (α subunit)	27280
Sandhoff	β–Hexosaminidase A and B (α and β subunit)	26880
Krabbe	Galactosylceramide β-galactactosidase	24520
Metachromatic leukodystrophy	Arylsulphatase A	25010
Mucolipidosis IV	Ganglioside neuraminidase	25265
Sulphatidosis	Multiple sulphatases	27220
Nieman-Pick:		
Type A, B	Sphingomyelinase	25720
Type C	Cholesterol esterification	25722
Cholesterol ester storage	Acid lipase	21500
Wolman	Acid lipase	27800
Pompe disease	α-glucosidase	23230
Glycoprotein metabolism		
Aspartylglucosaminuria	N-aspartyl-β glucosaminidase	20840
Fucosidosis	α-L-fucosidase	23000
α-Mannosidosis	α-D-mannosidase	24850
β-Mannosidosis	β-D-mannosidase	24851
Mucolipidosis 1	α-neuraminidase	25655
Schindler	α-N-acetylgalactosaminidase	10417
Mucopolysaccharidoses		
Hurler/Scheie(MPS 1)	α-Iduronidase	25280
Hunter(MPS 11)	Iduronate sulphate sulphatase	30990
Sanfilippo:		
A (MPS 111A)	Heparan sulphamidase	25290
B (MPS 111B)	N-acetyl α-glucosaminidase	25292
C (MPS 111C)	Acetyl-CoA: α-glucosaminide	25293

	N-acetlytransferase	
D (MPS 111D)	N-acetylglucosamine-6-sulphate sulphatase	25294
Morquio:		
A (MPS IVA)	N-acetylgalactosamine-6-sulphate sulphatase	25300
B (MPS IVB)	β-Galactosidase	25301
Maroteaux-Lamy (MPS VI)	Arylsulphatase B (N-acetyl galactosamine 4-sulphate sulphatase)	2532
Sly (MPS VII)	β–Glucuronidase	25322
2. Deficiencies of activating or protective factors.		
Galactosialidosis	Protective factor of β-galactosidase and sialidase	25654
Activator deficient Metachromatic leukodystrophy	Sphingolipid activator protein 1 (Saposin B)	24990
GM2 gangliosidosis AB variant	Activator protein of GM2-ganglioside	27275
3. Lysosomal membrane transport defect.		
Salla	Transport of sialic acid	26874
Cystinosis	Transport of cysteine	21980
Vit B12	Transport of vit B12	25100
4. Deficiencies of enzyme processing and targeting.		
Mucolipidosis II (I-cell)	N-acetylglucosaminylphospho -transferase	25250
Mucolipidosis III (Pseudo-Hurler)	N-acetylglucosaminylphospho -transferase	25260

Lysosomal storage diseases are clinically heterogenous, many ranging from very severe to benign forms. This reflects the large number of different disease-causing mutations in the same gene giving rise to the same lysosomal storage disease in different patients (Table 1.3). In some diseases a few mutations, often in a particular ethnic group, account for a large proportion of the patients, while many rare mutations account for the remainder. Compound heterozygotes with different disease-causing mutations on each allele have been found in many cases. In all the disorders many patients have as yet uncharacterised mutations. The best characterised disorders to date at the genetic level are Gaucher, Tay Sachs and metachromatic leukodystrophy.

1.1e. i). Gaucher disease.

Gaucher disease results from an inherited deficiency of acid β-glucocerebrosidase and is the most common lysosomal storage disease. Patients have been categorised into three clinical types: type 1 has a milder course with no neuronopathetic involvement whereas types 2 and 3 have acute and subacute neuronopathic involvement respectively. Type 1 has an increased frequency in the Ashkenazi Jewish population. Types 2 and 3 do not appear to have any ethnic predilection, but the largest number of type 3 patients has been reported from Norbotten, Sweden. Several point mutations and gene fusions (with the adjacent pseudogene) have so far been identified in the glucocerebrosidase gene, causing the deficiency. Several patients have unidentified mutations. In a study of 44 Gaucher patients by Theophilus et al. (1989b), 73% had at least one allele with an unknown mutation. The two most common mutations so far identified in Gaucher patients are: a missense mutation, 444Leu-Pro, in exon 10 which generates a Nci 1 site, and a missense mutation in exon 9, 370Asp-Ser. Most other mutations are rare or found only in one family. Some Gaucher alleles have been identified with as many as 7 mutations, which have a similar sequence to the corresponding region of the adjacent pseudogene (Latham et al., 1991). These complex alleles may result from gene conversion or nonhomologous recombination between the glucocerebrosidase gene and the pseudogene (Eyal et al., 1990; Latham et al., 1990; Hong et al.,1990). The 444Leu-Pro mutation is also present in the pseudogene.

Many attempts have been made to correlate clinical phenotype and genotype (Theophilus et al., 1989b; Zimran et al., 1989; Kolodny et al., 1990; Latham et al., 1990). Mutation 370Asp-Ser is always found in type 1 patients, homozygotes having a milder phenotype than compound heterozygotes. Homozygosity for the 444Leu-Pro mutation has until recently been associated with neuronopathic disease (Tsuji et al.,1987; Kolodny et al., 1990). However, five type 1 Japanese patients and one other case of type 1 have now been identified which are all homozygous for the 444Leu-Pro mutation (Masuno et al., 1990; Glew et al., 1991). The phenotype of specific mutations therefore appears to be modified by unknown variables. Correlation between genotype and phenotype will enable a more accurate prediction of clinical course and help in genetic counselling.

1.1e. ii). Tay Sachs disease.

Tay Sachs results from deficient activity of β-hexosaminidase A and has an increased frequency in the Ashkenazi Jewish population, French Canadians and the Moroccan Jewish population. Each ethnic group appears to have a prevalent mutation. Approximately 70% of Ashkenazi Jewish Tay Sachs patients have an insertion of 4 base pairs in exon 11, which alters the reading frame. Most of the remaining patients of this ethnic group have a splice site mutation at the 5' border of intron 12 . A 7.5 kb deletion predominates in French Canadian patients. Deletion of one phenylalanine codon appears to be a prevalent mutation in the Moroccan Jewish population. Some mutations have only been found in one family, while many patients have mutations as yet unidentified. A mutation responsible for late onset GM2 gangliosidosis has been identified as a G to A transition in exon 7, causing the α-subunit to associate poorly with the β-subunit. The low level of residual activity results in a milder phenotype with onset in adolescence or adulthood. A high proportion of point mutations in the Hex A gene are CG -> TG or CG -> CA transitions, consistent with the chemical model of mutation via methylation-mediated deamination.

Gaucher
Beta-D-glucocerebrosidase deficiency

1. A -> G AAC -> AGC	Asp370 -> Ser. Exon 9		Homozygotes are type 1. Frequent.	Tsuji et al. 1988
2. T -> C CTG -> CCG.	Leu444 -> Pro Exon 10. Inactive protein produced mutation in active site.	New Nci1 site.	Type 2 or 3 Gaucher. Frequent.	Tsuji et al. 1987. Dahl et al.1990.
3. C-> G CCC -> CGC	Pro415 -> Arg. Exon 9. Inactive protein produced.	New Hha1 site	Rare	Wigderson et al. 1989.
4. G -> A. CCG -> CAG	Arg119 -> Gly. Exon 5.	New BstN1 site.	Type 1. Rare.	Graves et al. 1988.
5	Fusion between 5' of functional gene and 3' of pseudogene.		Type 1.	Zimran et al. 1990.
6. G -> T GTG -> TTG.	Val 394 -> Leu. Exon 9.		1 type 1 1 type 3.	Theophilus et al. 1989.
7. G -> C. GAC -> CAC.	Asp409 -> His. Exon 9.		2 type 1 1 type 3.	Theophilus et al. 1989.
8. A -> T. GAC -> GTC.	Asp409 -> Val. Exon 9.		1 type 3.	Theophilus et al. 1989.

Table 1.3. Mutations in Lysosomal Storage Diseases.

9. G -> C GCT -> CCT.	Ala456 -> Pro. Exon 10.			Latham et al. 1990.
10. G -> A. 11. T -> G.	Gly 325->Arg. Cys342 -> Gly.		}1 compound heterozygote } type 2, non Jewish.	Eyal et al. 1991.
12. T -> A.	Phe255 -> Tyr.			Beutler et al. 1990.
13. C -> T.	Cys463 -> Arg.		1 allele.	Hong et al. 1990.
14. 5424C	Ser264 -> Thr. Exon 8.		Compound heterozygote with Leu444 -> Pro. Italian/Irish. Type 1.	Latham et al., 1991.
15. 5269T	Trp312 -> Cys Exon 8.		Compound heterozygote with Leu444 -> Pro. Italian. Severe bone disease.	Latham et al., 1991.
16. 5259T	Ala309 -> Val Exon 8.		Compound heterozygote Greek. Type 1	Latham et al., 1991.
17. 3170C	Lys157 -> Gln Exon 5.		Compound heterozygote with Leu444 -> Pro. Type 2.	Latham et al., 1991.
18. 5958T	Asp409 -> Val Exon 9.		Compound heterozygote with Leu444 -> Pro. Type 3.	Latham et al., 1991.
Complex alleles:				
3059T	Arg120 -> Trp	Exon 5	}Compound heterozygote	Latham et al., 1991.
3461C	Trp184 -> Arg	Exon 6	}with Asn370 -> Ser	" " " "
3475G	Asn188 -> Lys	Exon 6	}Severe splenomegaly.	" " "

Table 1.3. Mutations in Lysosomal Storage Diseases.

3483G	Val191 -> Gly	Exon 6	}Type 1.	" " " "
3497G	Ser196 -> Pro	Exon 6	}All 7 mutations found in	" " " "
3515A	Gly202 -> Arg	Exon 6	}pseudogene except	" " " "
3548A	Phe213 -> Ile	Exon 6	}Trp184 -> Arg.	" " " "
T -> C	Leu444 -> Pro.		}I complex	Hong et al. 1990
G -> C	Ala456-> Pro.		} allele, all 3 present	" " " "
G -> C	Val460 -> Val.		} in pseudogene.	" " " "

GM2 gangliosidosis.
a). Tay Sachs.
alpha subunit of beta-hexosaminidase .

1. G -> C.	5'boundary of intron 12. New splice site. Mature mRNA levels low.	New Dde site.	Classic Tay Sachs. 20-30% of Ashkenazi Jewish carriers.	Ohno et al. 1988. Myerowitz 1988 Arpaia et al. 1988.
2. TATC insertion.	4bp insertion in exon 11 leads to frameshift and premature termination 9n downstream. Mature mRNA absent.		Classic Tay Sachs. 70% of Ashkenazi Jewish carriers.	Myerowitz et al. 1988
3. 5' deletion.	7.6kb deletion of exon 1 and flanking sequences including the promoter No mRNA. Deletion between Alu sequences.		Prevalent among French Canadians.	Myerowitz et al. 1986.
4. G -> A.	Glu482 -> Lys.		Rare.	Proia et al.1982

Table 1.3. Mutations in Lysosomal Storage Diseases.

5. 1bp deletion.	Frameshift mutation in exon 13, leads to premature termination and loss of a hydrophobic stretch of 23 amino acids. Defective polypeptide does not exit ER.	Rare. 1 homozygote.	Lau et al. 1989.
6. G ->A.	Arg178 -> His. Exon 5. Enzyme hydrolyses neutral substrate but not GM2 gangliosides (B1 variant).	2 homozygous Portuguese. 1 homozygous French.	Ohno et al. 1988. Alki et al. 1991.
7. G-> A.	Last nucleotide of exon 5 Aberrant mRNA processing.	Rare. 1 homozygous Tunisian. Late infantile.	Alki et al. 1990.
8. G -> A.	Arg504 -> His. Exon 13. Alpha subunit fails to associate with beta subunit.	1 homozygote, 1 heterozygote. Juvenile form. 1 heterozygous Lebanese.	Paw et al. 1990. Boustany et al. 1991.
9. G -> C.	Trp420 -> Cys. Exon 11. mRNA present. Kpn1 site lost.	One heterozygote. Infantile form.	Tanaka et al. 1990a.
10. 3bp deletion.	In frame deletion of one of two Phe codons TCC 304 or 305. Exon 8.	1 Moroccan Jewish and 3 unrelated carriers. 3 French compound heterozygotes.	Navon et al. 1991 Alki et al 1991.
11. G -> A.	Arg499 -> His Exon 13. Protein retained in ER.	1 heterozygote juvenile form.	Paw et al. 1990

Table 1.3. Mutations in Lysosomal Storage Diseases.

12. C -> T.	Ser210 -> Phe. Exon 6, mRNA produced.	2 homozygous brothers. Algerian.	Alki et al. 1991.
13. C -> T.	Arg504 -> Cys. Exon 13. mRNA produced.	1 homozygous Algerian 1 compound heterozygous French.	Alki et al. 1991.
14. C -> T.	Arg137 -> Stop codon. Exon 3. Low level mRNA.	1 French compound heterozygote.	Alki et al. 1991.
15. C ->T.	Arg393 -> Stop codon. Exon 11. Low level mRNA.	1 French compound heterozygote.	Alki et al. 1991.
16. G -> A.	Donor splice site mutation First nucleotide of of intron 2.	1 French compound heterozygote.	Alki et al. 1991.
17. G -> A.	Donor splice site mutation. Fifth nucleotide of intron 4.	1 French compound heterozygote..	Alki et al. 1991.
18. G -> T	AG acceptor splice site preceding exon 5.	1 homozygote, 1 compound heterozygote, both with classic phenotype, both American blacks.	Mules et al. 1991.
19. C -> T.	Arg178 -> Cys. *Exon 5, B1 variant.*		Tanaka et al. 1990b.
20. G -> A.	Arg 170 -> Gln	1 heterozygous Japanese.	Nakano et al. 1990.
Late onset gangliosidosis.			
1. G -> A.	Gly269 -> Ser. Exon 7. Altered alpha subunit associates poorly with beta subunit.	1 compound heterozygote.	Navon et al. 1989.

Table 1.3. Mutations in Lysosomal Storage Diseases.

GM gangliosidosis 11 (Sandhoff).
Beta subunit of beta hexosaminidase.

1. G -> A.	Generates a splice site 26bp from 3' terminus of intron 12, resulting in the insertion of 24bp in the mRNA, and 8 amino acids in the protein.		4 compound heterozygotes, including 3 Sandhoff patients and one asymptomatic individual. 1 Japanese ,juvenile form.	Nakano et al. 1989. Mitsuo et al. 1990.
2. 50kb deletion.	5' end of gene.		2 'classic' Sandhoff patients compound heterozygotes.	Bikker et al. 1989.
3.Duplication.	Duplication of region around the junction of intron 13/exon 14 Generates an alternate splice site causing an in frame insertion of 18n in mRNA.		One asymptomatic heterozygote.	Dlott et al. 1990.
4. 16kb deletion.	Deletion includes promoter and exons 1-5, recombination between 2 Alu1 sequences.		2 homozygotes infantile form, 4 compound heterozygotes, infantile juvenile or adult onset.	Neote et al. 1990.

Fabry.
Alpha-D-galactosidase deficiency.

1. C -> T.	Arg356 -> Trp. Exon 7.	Msp 1 site lost.		Bernstein et al. 1989.
2. Deletions.	Deletions found in 5 patients with different break points, ranging in size from 0.4-5.5kb.			Bernstein et al. 1989.

Table 1.3. Mutations in Lysosomal Storage Diseases.

3. Duplication ~ 8kb duplicated, exons 2-6.				Bernstein et al. 1989.
4. C -> T	Pro -> Ser			Koide et al. 1990
5. G -> A.	Trp44 -> stop codon Exon 1.	New Nhe1 site.	1 Japanese hemizygote.	Sakubara et al. 1990.
6. G -> A	Arg 301-> Gln. Exon 6. Significant residual activity.		1 Japanese hemizygote, late onset.	Sakubara et al. 1990.
7. 13bp deletion.	Exon 1.			Ishii et al. 1991.
Fucosidosis. Alpha-L-fucosidase deficiency.				
1. C -> G.	Gln -> stop codon. Exon 8. Premature termination 120bp upstream of normal stop codon.	EcoR1 site lost.	5 homozygous patients, 2 compound heterozygous siblings. 2 Cuban, 2 French, 3 Italian.	Kretz et al. 1989.
2. Deletion.	3' end of gene, >2kb.		2 Algerian siblings.	Willems et al. in press.
Aspartylglucosaminuria. Glycosylasparaginase deficiency.				
1. G -> C.	Cys163 -> Ser. mRNA present .Defective post translational processing .	EcoR1 site created.	3 homozygous Finnish patients. 20 Finnish patients.	Fisher et al. 1991. Ikonen et al. 1991.
2. G -> A	Gly302 -> Arg		1 homozygous Turkish patient	Ikonen et al. 1991.

Table 1.3. Mutations in Lysosomal Storage Diseases.

3. T -> C	Cys306 -> Arg	1 homozygous white American	Ikonen et al. 1991.
4. G -> A	Gly60 -> Asp	1 homozygous German	Ikonen et al. 1991.
5. C -> T	Ala101 -> Val	1 homozygous Italian	Ikonen et al. 1991.
6. Deletion of T 336	Frameshift, premature termination.	1 homozygous Dutch	Ikonen et al. 1991.
7. 7nt deletion (nt102-108)		1 compound heterozygote with Ala101 -> Val, English	Ikonen et al. 1991.
8.Insertion of T after nt800	Frameshift.	1 homozygous Spanish American.	Ikonen et al. 1991.
9.Insertion of 6nt after nt127.	Insertion of Asp, Ala.	1 homozygous Tunisian.	Ikonen et al. 1991.
10. G -> T	Position +1 of splice donor site. Skipping of exon (nt807-940)		Ikonen et al. 1991.
Hunter.			
Alpha-L-Iduronate sulphate sulphatase deficiency.			
1. Deletions.	7/23 patients with gross structural alterations, including 2 with gene totally deleted.		Wilson et al., 1990

Table 1.3. Mutations in Lysosomal Storage Diseases.

Sly. (MPS V11) Beta-D-glucuronidase deficiency				
1. C -> T.	Ala619 -> Val. mRNA levels normal.	Fnu4H1 site lost.	1 homozygous Japanese adult.	Tomatsu et al. 1991.
2. C -> T.	Arg382 -> Cys. mRNA levels normal.		1 homozygous Japanese 7 yr old.	Tomatsu et al. 1991.
Schindler. Alpha-N-acetylgalactosaminidase deficiency.				
1. G ->A.	Glu325 -> Lys. mRNA present, unstable protein.		2 homozygous brothers.	Wang et al. 1990.
Nieman Pick. Acid sphingomyelinase deficiency.				
1. G -> T.	Arg496 -> Leu.			Levran et al. 1991.
Metachromatic leukodystrophy. Arylsulphatase A deficiency.				
1. G -> A.	Donor splice site mutation in intron 2 . Unstable mRNA.		Homozygotes have late infantile phenotype. Approx. 25% of ASA alleles in patients.	Polten et al. 1991.
2. C -> T.	Pro426 -> Leu. Protein with reduced half life.		Homozygotes have adult onset phenotype. Approx. 25% of ASA alleles. Compound heterozygotes with splice site mutation have juvenile phenotype.	Polten et al. 1991.

Table 1.3. Mutations in Lysosomal Storage Diseases.

3. G -> A.	Gly99 -> Asp. Exon 2.	Adult type.	Kondo et al. 1991.
4. 11bp deletion.	Exon 8. mRNA produced.	Late infantile compound heterozygote.	Bohne et al. 1991.
Activator deficient metachromatic leukodystrophy. Saposin B deficiency.			
1. C -> T.	Thr -> Ile		Rafi et al., 1990
2. G -> C.	Cys241 -> Ser. mRNA present.	1 compound heterozygote.	Holtschmidt et al. 1991.
3. C -> A.	Creates a new 3' splice junction. 33bp insertion.	1 patient.	Zhang et al. 1991.
Gaucher variant. Saposin C deficiency.			
1. G -> T.	Cys385 -> Phe.		Schnabel et al. 1991.

Table 1.3. Mutations in Lysosomal Storage Diseases.

1.1e. iii). Metachromatic Leukodystrophy.

Metachromatic leukodystrophy results from a deficiency of arylsulphatase A or the sulfatide activator protein. Two mutations in the arylsulphatase A gene together account for approximately 50% of metachromatic leukodystrophy alleles: one splice site mutation and one missense mutation. A correlation has been found between aryl sulphatase A genotype and clinical phenotype. Homozygotes for the splice site mutation have the late infantile form of disease, homozygotes for the missense mutation have the adult or the juvenile form, whereas compound heterozygotes for both mutations have the juvenile phenotype (Polten et al, 1991).

Two point mutations and one insertion mutation have also been found in the gene coding for saposin B which cause activator deficient metachromatic leukodystrophy (Table 1.3).

1.1f. Pseudodeficiency of a lysosomal enzyme.

Deficient activity of a lysosomal enzyme has been found in some clinically normal individuals. Low activity polymorphisms have been found for several lysosomal enzymes including α-L-iduronidase, (Whitley et al., 1987), aryl sulphatase A (Dubois et al., 1977), β-hexosaminidase A (Vidgoff et al., 1973), β-hexosaminidase A and B (Dreyfus et al., 1975), galactocerebrosidase (Wenger et al., 1976), and α-galactosidase A (Bach et al., 1982). Low fucosidase activities have been found in plasma and fibroblasts, but not leukocytes, of individuals with a normal phenotype (Ng et al., 1973; Van Elsen et al., 1983). Low activities of lysosomal enzymes appear to be compatible with normal substrate degradation, if these activities exceed a certain threshold, which may be lower than 10% of normal activity. Two A - G transitions, one resulting in loss of a polyadenylation signal, have been found in the aryl sulphatase A (ASA) pseudodeficiency allele and result in very low ASA activity, making it difficult to distinguish between the pseudodeficiency and metachromatic leukodystrophy on the basis of enzyme activity. The low residual activity found in pseudodeficient individuals must however be sufficient to prevent storage of cerebroside sulfate and demyelination.

Pseudodeficiency of lysosomal enzymes complicates enzymatic diagnosis of the

disease considerably and is a problem in genetic counselling. It has been the cause of some misdiagnoses (Kappler et al., 1991). If, however, the mutation causing the pseudodeficiency or the disease is known, DNA based tests can be used reliably for the detection of patients or carriers. Gieselmann (1991) has developed a test for the ASA pseudodeficiency allele using a polymerase chain reaction-based assay using pairs of primers that amplify either the pseudodeficiency allele or the ASA allele.

1.1g. Glycoprotein breakdown.

Mammalian tissues contain glycoproteins with two different types of protein-linked glycans with distinct synthetic pathways and structures. 'O'-linked glycans are synthesised by the sequential transfer of single sugars from sugar nucleotides to the growing oligosaccharide chain. Oligosaccharides are linked to the protein by an 'O'-glycosidic linkage of N-acetylgalactosamine (GalNAc) to serine or threonine (eg. blood group substances). 'N'-linked glycans are synthesised via a dolichol pyrophosphate-linked intermediate. The oligosaccharide precursor is transferred from the dolichol pyrophosphate to the amide nitrogen of an asparagine residue in the protein to form a glycosidic linkage between a N-acetylglucosamine at the non reducing terminal of the precursor oligosaccharide and the asparagine. Following trimming and elongation steps, 'high mannose', 'hybrid' and 'complex' types of oligosaccharide chains are formed.

The protein backbone of glycoproteins is degraded by a mixture of lysosomal endo- and exo-peptidases to yield glycoasparagines. Asparagine-linked glycans are broken down in a bidirectional manner by the concerted action of exoglycosidases at the non reducing terminal and by aspartyl N acetyl glucosaminidase and endohexosaminidase at the reducing end (Brassart et al., 1987; Aronson et al., 1989; fig. 1.1).

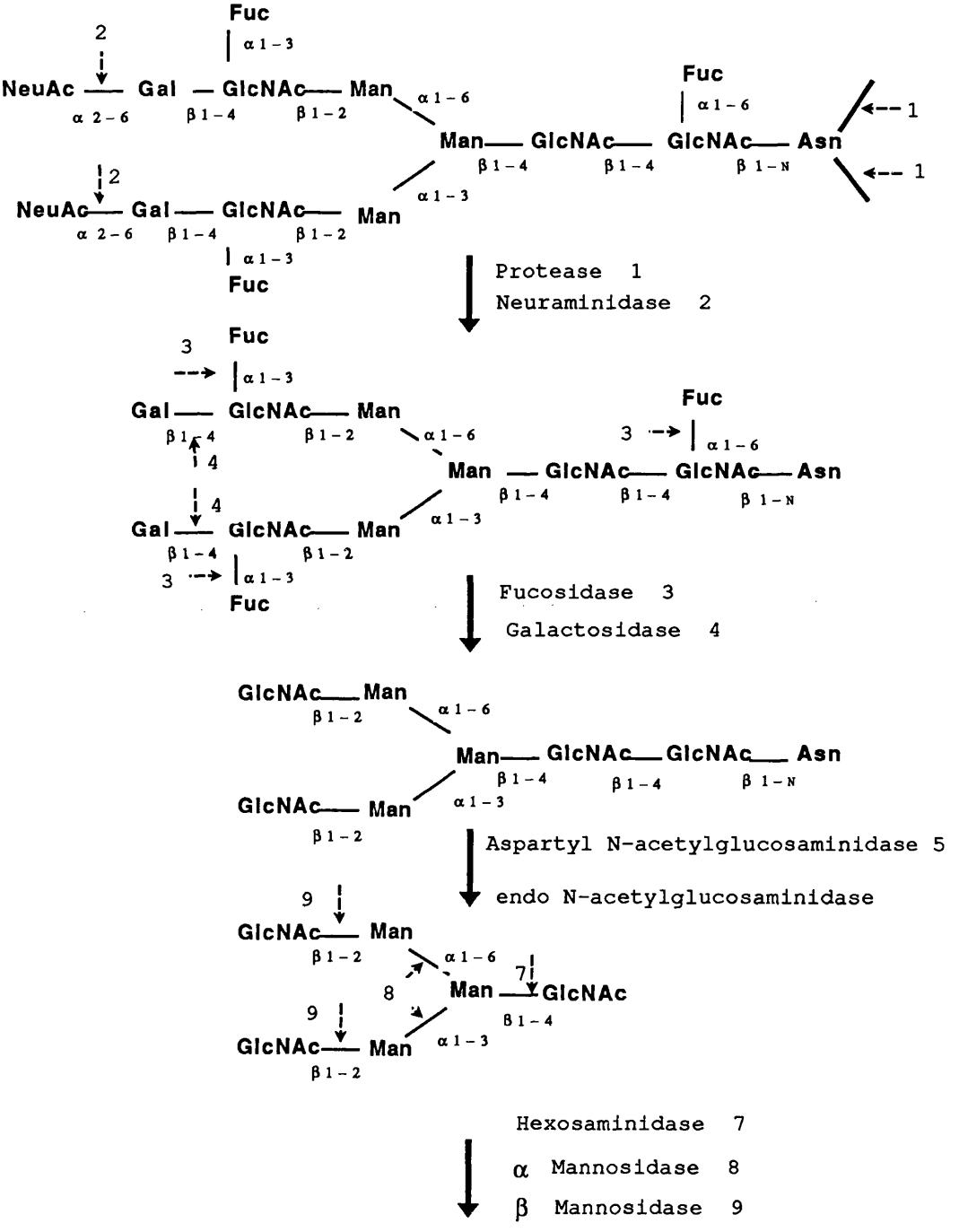


Fig.1.1. Lysosomal degradation of a representative complex asparagine-linked glycan in a glycoprotein.

1.2. α-L-fucosidase.

1.2a. Occurrence.

α–L-fucosidase (EC 3.2.1.51) activity has been found in the lysosomes of all human tissues investigated, including liver, fibroblasts, leucocytes, brain, spleen, kidney and placenta; and in plasma (Robinson and Thorpe, 1973; Zielke et al., 1972; Matsuda et al., 1973; Chien and Dawson, 1980; Wiederschain et al., 1971; Alhadeff and Janowsky, 1978). Expression of the gene appears to commence early in development. α-L-fucosidase is active in liver of human embryos at 5 to 7 weeks gestation, and is found in amniotic fluid (Wiederschain et al., 1971). Serum fucosidase activity is at its highest 15 days after birth and decreases from this level for approximately 1 year, after which it remains constant (Vaysse et al., 1990).

Fucosidase activity has been reported in rat liver Golgi, rough and smooth endoplasmic reticulum and plasma membrane, as well as lysosomes. Nuck et al. (1987) were able to distinguish between the activities from different subcellular structures using the fucosidase inhibitor, deoxyfuconojirimycin. Tauber et al. (1985) found that degradation of the polypeptide moiety of the plasma membrane glycoprotein Gp120 was inhibited by treatment of rats with chloroquine, an inhibitor of lysosomal enzymes. However, this treatment did not affect the half life of fucose bound to the glycoprotein suggesting that the fucose is removed prior to segregation of the glycoprotein to the lysosomes. The authors suggest that the fucose may be removed from the glycoprotein in the plasma membrane, by glycosidases in the serum. Fucosidase activity other than in lysosomes or plasma has not been reported in humans.

1.2b. Kinetic properties.

1.2b. i). pH-dependence of activity.

α-L-fucosidase has a broad activity-pH profile with an optimum between pH 5.0 - 5.5, and a shoulder of activity at pH 6.0.

1.2b. ii). Michaelis-Menten constant.

Km values have been reported between 0.055mM (Willems et al., 1981) and 0.52

mM (Alhadeff and Janowsky, 1978) for α-L-fucosidase acting on the synthetic substrates 4 methyl umbelliferyl-α-L-fucoside (4MU) or *p*-nitrophenyl-α– L-fucoside (PNP) (Dicioccio et al., 1982; Troost et al., 1976).

1.2b. iii). The active site.

Radiolabelling with I ^{3}H conduritol trans epoxide has shown that four active sites are present per tetrameric molecule (White et al., 1987). Kinetic and inhibitor studies suggest that at least two carboxyl groups are present whose ionisation state is important for activity, at least one being in the active site. Proton transfer appears to be the rate-limiting step in the enzyme reaction. A general acid base catalytic mechanism has been proposed by White et al.,(1987).

Deoxyfuconojirimycin is a specific competitive inhibitor of α-L-fucosidase (Ki $1x10^{-8}$ M). The pH-dependence of inhibition by deoxyfuconojirimycin and its analogues suggests that inhibition results from the formation of an ion pair between the protonated inhibitor and a carboxylate group in the active site of the enzyme (Winchester et al., 1990). This supports the mechanism of action proposed by White et al (1987).

1.2b. iv). Substate specificity.

α–L- fucosidase is a lysosomal exoglycosidase which cleaves fucose from the non reducing end of oligosaccharides, glycoproteins and glycolipids. The enzyme shows specificity for Fuc α1-2 Gal, Fuc α1-3 GlcNAc, Fuc α1-4 GlcNAc, and Fuc α1-6 GlcNAc linkages. The rate of hydrolysis of these linkages is dependent on the structure of the rest of the substrate molecule. Specificity experiments using purified serum enzyme and synthetic substrates have shown, for example, that the rate of hydrolysis of the aryl derivatives, Fuc α1-2 Gal β1-0$C_6H_4NO_2$, Fuc α1-3 GlcNAc β1-OC_6H_5 and Fuc α1-4 GlcNAc β1-OC_6H_5 were hydrolysed 5, 11 and 53 foldfaster than their respective simple disaccharides (Fuc α1-2 Gal, Fuc α1-3 GlcNAc and Fuc α1-4 GlcNAc). This was not the case for Fuc α1-6 GlcNAc and its aryl derivative, however, both being hydrolysed at a slow rate. Branched groups attached to the anomeric position of galactose in the disaccharide Fuc α1-2 Gal appear to provide steric hindrance and are hydrolysed at a

much slower rate than equivalent unbranched groups (Dioccio et al., 1982). Thorpe and Oates., (1978) investigated the ability of purified α-L-fucosidase to cleave naturally occurring substrates. Fucosidase activity decreased as the molecular weight of the oligosaccharide increased. Fucose was cleaved from He and Le blood group oligosaccharrides.

As α-L-fucosidase hydrolyses the fluorigenic substrate, 4 methyl umbelliferyl-α-L-fucoside (4MU) and the colorigenic substrate, *p*-nitrophenyl -α-L-fucoside (PNP), these synthetic substrates are conveniently used in routine fucosidase assays.

1.2c. Structure.

1.2c. i). Isoenzymes.

α-L- fucosidase from humans and many other vertebrates can be resolved into several forms by starch gel electrophoresis or isoelectric focusing. As many as 7 major forms have been resolved with isoelectric points ranging between pI 4 and 7. Four major peaks of pI 6.3, 5.9, 5.6 and 5.4 were resolved following isoelectric focusing of human liver α-L-fucosidase (Thorpe and Robinson, 1975). A similar heterogeneity of isoelectric forms has been demonstrated in several other tissues including leukocytes, urine, placenta, spleen, and kidney (Turner et al., 1974; Beyer and Wiederschain, 1982; Gill and Sutton, 1984). Each isoform has a stable and separate identity. When individual isoforms, separated on thin layers of Sephadex G-50, are isolated, resuspended, and refocused on polyacrylamide, each remains a single band and retains the same pI (Thorpe and Robinson, 1975). Some minor differences in physical properties have been observed between isoforms, including slight differences in Km and pH (Alhadeff et al., 1978b). Thermostability has been shown to increase with isoforms of increasing acidity (Alhadeff et al., 1980a). All the isoenzymes are precipitated with the same antibody and migrate as two bands (representing the two subunits) on a polyacrylamide gel under dissociating conditions.

Following treatment with neuraminidase the liver α-L-fucosidase minor bands of low pI are lost and novel forms of higher pI appear, suggesting that the difference in mobility of some of the minor forms is due to sialic acid content. A minor shift to less anodal forms has also been observed for other tissues, following neuraminidase

treatment. Attachment of sialic acid to neuraminidase-treated liver α–L-fucosidase using sialyl transferase results in regeneration of the more acidic isoenzymes with the origional pI and in nearly the same proportions as before neuraminidase treatment (Alhadeff et al., 1978b). This implies that isoenzymes are related at least in part by sialic acid content.

The isoelectric focusing profile of serum α-L- fucosidase is different from that of tissue forms. It is more diffuse and there are more acidic forms of pI 3.8 to 6.8 (Alhadeff and Janowsky, 1978). Neuraminidase treatment results in loss of the acidic forms of pI 3.8 to 6.8 and an increase in the forms with a pI of 6.0 to 7.2, producing a diffuse band migrating in the same region as the cellular forms. Most of the difference in isoelectric focusing profile between the serum and tissue forms is therefore probably due to a higher sialic acid content of the serum enzyme. Twice as much sialic acid has been found in the enzyme purified from serum as that from liver (Alhadeff and Janowsky, 1978).

Variations in quantities and ratios of the multiple forms of α-L-fucosidase have been observed in enzyme present in the same tissues of different individuals. Turner et al. (1975b) distinguished three groups of isoelectric focusing patterns of α-L-fucosidase with characteristic profiles after neuraminidase treatment. Inheritance of the three phenotypes is consistent with segregation of two codominant alleles at a single autosomal locus. The frequency of the two alleles Fu 1 and Fu 2, has been determined in various populations . In the U.K. (n=109) and New York (n=194) the frequency of Fu2 was 0.25 (Corney et al., 1977, Turner et al., 1975a). Study of inheritance of the polymorphism in fucosidosis families, (Hirschhorn et al., 1976; Turner et al., 1975b) provided evidence for a third allele, Fu 0, at the same locus, which is responsible for fucosidosis when homozygous. Fu 1, Fu 2 and the fucosidosis allele(s) are therefore allelic and have been mapped to chromosome 1 p34 in close linkage disequilibrium with the Rhesus blood group (Corney et al., 1977; Carritt et al., 1982). Strong linkage disequilibrium has also been shown between the electrophoretic polymorphism and a Pvu11 restriction fragment length polymorphism at FUC A1 (Darby et al.,1988).

Tummler et al. (1984) found a correlation between expression of the tissue polymorphism and the level of synthesis of α-L-fucosidase during growth of lymphoid

cell lines. In cell lines derived from individuals homozygous for Fu1 allele, α-L-fucosidase synthesis was proportional to total protein synthesis, whereas for Fu2 homo- or heterozygotes, α–L- fucosidase synthesis increased relative to total protein during log phase growth. However, Dioccio et al. (1988) did not corroborate these results, finding synthesis of the enzyme being proportional to that of total protein in 7 cell lines with one or more Fu2 allele(s).

The various isoforms therefore appear to be encoded by the same structural locus, FucA1, but differ in N glycosylation, particularly sialic acid content.

1.2c. ii). Molecular structure.

Human α-L-fucosidase has been separated into two forms by a number of different techniques including gel filtration, affinity chromatography, ion-exchange chromatography and chromatofocusing. Robinson and Thorpe (1973) separated a high molecular weight form of 200,000 Da (Fuc 1), from a low molecular weight form of 50,000 Da (Fuc 2) by gel filtration on Sephadex G-200. Fuc 1 and Fuc 2 have identical isoelectric profiles (Thorpe and Robinson, 1975). The two forms are interconvertible. When Fuc 2 was re-analysed by gel filtration, activity was recovered as a mixture of forms 1 and 2. A mixture of 1 and 2 was recovered almost entirely as Fuc 1 (Thorpe and Robinson, 1978). Interconversion is dependent on pH and ionic strength, with most of the enzyme being in the form of Fuc 1 at pH 5.0 and form 2 at pH 6.8. A shift to form 2 was seen when the ionic strength was increased by changing from 5mM to 100mM sodium chloride. These results support a monomer-polymer relationship between the two forms with Fuc 1 being an aggregated form of Fuc 2, which consists of individual subunits of approximately 50,000.

Chien and Dawson (1980) subsequently separated two forms of α-L-fucosidase on the basis of binding (Fuc 1) or failure to bind (Fuc 2) to a fucosamine agarose affinity column. Fuc 1 had a molecular weight of 100,000, as shown by gel filtration on Sephadex G-200 and a molecular weight of 50,000 following denaturing SDS polyacrylamide gel

electrophoresis, whereas Fuc 2 had a molecular weight of 50,000 after both procedures. The two forms have several different physical properties, in addition to their different affinity for the fucosamine matrix. In view of these results Chien and Dawson propose that the 50,000 Da subunit of the bound fraction (Fuc 1) is not identical to the 50,000 Da unbound fraction (Fuc 2). The aggregated form may be made up of one type of subunit only, or a combination of the two.

Alhadeff and Andrews-Smith (1979) used the same fucosamine affinity column as Chien and Dawson to purify the enzyme prior to separation of two forms. By purifying the enzyme in this way, the unbound fraction, (Fuc 2) would presumably have been lost. However, affinity purified enzyme was separated into different subunits of 54,000 and 59,000 following reduction, S- carboxymethylation and electrophoresis in polyacrylamide gels under denaturing conditions. This suggests that the aggregated form is indeed made up of two different subunits. Laury-Kleintop et al. (1987) also resolved two fucosidase subunits with Mr's of 51,000 and 56,000 by electrophoresis under denaturing conditions.

1.2c. iii). Biosynthesis and post translational modification.

Like other lysosomal enzymes, α–L-fucosidase is a glycoprotein and has a carbohydrate content of approximately 7% (Alhadeff and Freeze, 1977). Nmr studies have shown that oligomannoside and N acetyl lactosamine type groups are present in a ratio of approximately 5 to 4 (Argade et al., 1988). Beem et al. (1987) reported a ratio of 3:1.

Pulse chase experiments have shown that the nascent fucosidase polypeptide is rapidly converted to a form of 58,000 Da and subsequently (after 21 hours of chase) to a 60,000 Da intracellular form and a 62,000 Da extracellular end product. This processing involves addition of carbohydrate side chains to a polypeptide precursor. Differential digestion with endoglycosidase H and N-glycanase suggests that each form is a glycoprotein with a common polypeptide of 52,000 Da with different carbohydrate moities attached. The carbohydrate moities of the 58,000 Da intracellular enzyme and the 62,000 Da extracellular form are high mannose and complex type respectively. The 60,000 Da intracellular form has a combination of both types of oligosaccharide. Based on the increase in molecular weight four carbohydrate chains are attached per 52,000 Da

molecular weight polypeptide (Dioccio et al., 1988). This is consistent with the cDNA sequence which has at least four potential glycosylation sites (Fukushima et al., 1985). Other lysosomal enzymes are synthesised as high molecular weight precursors which are proteolytically cleaved during post translational processing (Kornfeld et al., 1986). This does not appear to be the case for α-L-fucosidase.

Johnson and Dawson (1985) reported proteolytic processing of a 53,000 Da precursor to a 50.000 Da product. The 53,000 Da protein was found following NH_4Cl treatment of the fibroblasts.

1.2c. iv). Summary of the structure of fucosidase.

Fucosidase appears to be made up of two different subunits of 51kDa and 56kDa which can be resolved by electrophoresis on polyacrylamide under denaturing conditions. These two subunits are encoded by the same gene and may differ in glycosylation and/or proteolytic processing, or possibly alternative splicing of the mRNA. Under native conditions α-L-fucosidase has a molecular weight of approximately 200kDa, which suggests that a tetramer of these subunits exists under native conditions. The five different isoforms separated under native conditions according to size or charge may result from different combinations of the 51kDa and 56kDa subunits (ie. 4A, 3A1B, 2A2B, 1A3B, 4B). Fuc 1, the aggregated form of approximately 200kDa, probably represents tetramers of the 51kDa and 56kDa subunits. The different isoforms would not have been resolved under the conditions in which Fuc 1 and 2 were separated. Fuc 1 may partially dissociate under native conditions to form Fuc 2, which would represent individual 51kDa and 56kDa subunits. The aggregated form is more active and is probably the prevalent form in vivo (C.Barker 1987).

The Fu1 and Fu2 polymorphism, with two different patterns of isoenzymes has been linked to the fucosidase gene. The 51kDa and 56kDa subunits may have been isolated from one individual of one phenotype (Fu1 or Fu2) and the other phenotype may have subunits of a different size. This could result from a polymorphism in the amino acid sequence which would for example change a glycosylation site. This would affect both subunits. Alternatively the Fu1 and 2 polymorphism may affect the proportion of 51kDa and 56kDa subunits.

1.2d. Low plasma activity polymorphism.

A small proportion of the phenotypically normal population has a low plasma α-L- fucosidase activity (Ng et al., 1973), with activities between 10 and 30 % of the control mean. Segregation ratios in family studies have shown that the low activity polymorphism is inherited in an autosomal recessive manner (Eiberg et al., 1984), low activity 'variants' being homozygous for the low activity allele, a. The allele frequency for the variant gene is 0.28 in Italian and Danish populations, (Gatti et al., 1979; Eiberg et al., 1984) 0.29 in the U.K, (Playfair et al., 1976), 0.33 in Belgium (Willems et al., 1981) and 0.25 in Canada (Wood et al., 1979). Approximately 8% of the normal population are homozygous for the low activity allele and 52% homozygous for the normal gene. Linkage of the low plasma activity polymorphism to the plasminogen locus has been demonstrated, which has subsequently been assigned to chromosome 6 (Eiberg et al., 1984; Murray et al., 1987).

Individuals who are homozygous for the low activity allele also have reduced α-L- fucosidase activity in cultured fibroblasts. Low plasma activity variants have fibroblast fucosidase activities less than 50 % of the mean control at each stage of the cell cycle (Van Elsen et al., 1983). Fibroblast extracellular activity decreases proportionally to fibroblast intracellular activity, so that the ratio of intracellular and extracellular activity is the same for variants and non variants.

In contrast to plasma and fibroblasts, the leukocyte fucosidase activity of low plasma activity variants and controls is barely distinguishable, although Gatti et al., (1979) claimed to have found a statistically significant difference. Assay of leukocyte activity is therefore the most reliable method for detection of carriers of fucosidosis.

Ovarian cancer patients are 3 fold more likely to have low plasma α-L-fucosidase activity than healthy females. This is not a result of the disease but is genetically determined, as in the general population. The low plasma activity polymorphism may therefore be a hereditary condition associated with an increased risk of ovarian cancer (Barlow et al., 1981).

The plasma α–L-fucosidase from individuals with low activity can be distinguished from that of individuals with high plasma activity by its relatively lower activity and greater heat lability at acidic pH (Willems et al., 1981). α-L-fucosidase exists in two forms (see 1.2.c.ii). The aggregated form is prevalent at acidic pH and is heat stable whereas the monomeric heat labile form is prevalent at pH 8. In both variant and non-variant plasma and fibroblasts, the monomeric form predominates at pH 8. At pH 5 non-variant α-L-fucosidase occurs in the heat stable aggregated form whereas most of the enzyme remains in the heat labile less active monomeric form in variants. This explains the observation that the variant enzyme has a lower activity and is heat labile at acidic pH. These results were interpreted as indicating a structural difference between the enzyme from variants and non variants. DiCioccio et al. (1983) subsequently found that the conversion of monomeric to aggregated form is also dependent on enzyme concentration. Fucosidase was purified from serum of non variants and variants. With increasing dilutions of the enzyme from high activity serum the enzyme was more heat labile and less active at pH 5. At equivalent units of enzyme the proportions of heat labile and stable forms at pH 5 were the same for high or low activity enzyme.

Therefore the two distinguishing features of the variant plasma α-L-fucosidase heat lability and non aggregation can be explained simply by the lower concentration of enzyme found in variants which would decrease the conversion of monomeric to aggregated form due to a lower pH. They are not a result of structural differences.

Using an ELISA method, DiCioccio et al. (1986) showed that the low activity variants have a low concentration of fucosidase protein in serum compared to non variants. A statistical correlation was found between the amount of α–L-fucosidase protein and enzyme activity of low and high activity plasma. Serum from variants and non variants had similar α-L- fucosidase specific activity at a mean of 11,002 + 1051 U/mg. Variants therefore have low amounts of enzyme of the same specific activity as non variants.

It therefore appears that the plasma low activity polymorphism is the result of

decreased amounts of fucosidase with the same structural and kinetic properties, apparently determined by a gene on chromosome 6. The molecular basis for the polymorphism is not known. It could result from lower levels of gene expression, although this seems unlikely as different quantities are present in serum, fibroblasts and leukocytes. Differences in post translational modification may result in α-L-fucosidase which has reduced stability in plasma, and to a lesser extent, in fibroblasts. Plasma glycoproteins with galactose as the penultimate sugar are removed from plasma by specific receptors when the terminal sialic acid is lost. Dreyfus and Poenaru (1977) propose that fucosidase in low plasma activity variants may have a faster clearance rate from plasma by this mechanism, perhaps due to a difference in the carbohydrate moiety of the enzyme. This would not explain the concomitant reduction in fibroblast activity however.

Pseudo deficiency of several other lysosomal enzymes has been reported including aryl sulphatase A (ASA), α-galactosidase A, β-hexosaminidase A , iduronidase and galactocerebrosidase (Dubois et al., 1977; Bach et al., 1982; Vidgoff et al., 1973; Whitley et al., 1987; Wenger et al., 1976) but a tissue-specific deficiency has only been observed for fucosidase. The mutation causing ASA pseudodeficiency is in the gene coding for the enzyme, unlike the polymorphism for fucosidase plasma activity. Variation in a fucosidase activity has been found between inbred mouse strains. The polymorphism is expressed in every tissue studied, in contrast to the human case (Johnson and Hong, 1986).

1.3. Fucosidosis

1.3a.Incidence and genetics.

Fucosidosis has a worldwide distribution and a very low incidence. In a recent review, involving literature surveys and an international questionnaire, only 77 cases were identified (Willems et al., 1991) in eighteen different countries. Fucosidosis patients have been identified in North and South America, Africa, Western Europe, and Japan. A relatively higher frequency of patients has been found in Italian populations and the Mexican Indian population of Colorado and New Mexico. Out of the 77 patients identified in the review, twenty were of Italian descent and eight were Mexican Indian.

Romeo et al. (1977) proposed that a founder effect could explain the increased incidence of fucosidosis in Italian populations. However it is now evident that several different mutations cause fucosidosis. The observed increased incidence in these two ethnic groups may be partly due to increased screening in these areas.

Pedigree studies have shown that fucosidosis is inherited in an autosomal recessive manner. Parents of fucosidosis patients are phenotypically normal, and have intermediate α-L- fucosidase activities. Recurrence of fucosidosis in siblings of index cases was 32% in 45 sibships (Willems et al., 1991). This is not significantly different from the 25% expected for autosomal recessive diseases. Parental consanguity has been reported in 18 out of 45 (40%) of fucosidosis families, so many of the patients are probably homozygous for the same mutation.

1.3b. Clinical features.

1.3b. i) Phenotype.

The clinical phenotype of fucosidosis was first described by Durand et al. (1966) in two Italian siblings with progressive mental retardation, leading to decerebrate rigidity and death before five years. A deficiency of α-L-fucosidase in these two siblings and an additional patient of similar phenotype was subsequently found by Van Hoof and Hers

(1968). A different phenotype of milder course with angiokeratoma and survival to over 20 years was later described by Patel et al. (1972).

The most prevalent presenting symptom found in a study of 77 fucosidosis patients (Willems et al., 1991) was psychomotor delay with a mean age of presentation of 1.2 +0.8 years. Patients then show a progressive neurological deterioration with loss of mental and motor abilities. Mental retardation was present in all patients on whom enough data were available, and was usually severe. The main clinical features consist of : progressive mental (95%) and motor (87%) deterioration, coarse facies (79%), growth retardation (78%), recurrent infections (78%), dystosis multiplex (58%), angiokeratoma corporis diffusum (52%), visceromegaly (44%), and seizures (38%) (Willems et al., 1991).

Considerable clinical heterogeneity has been observed in fucosidosis patients. Because of the wide range of different phenotypes Kousseff et al.(1973) divided the disorder into two types according to severity. Type 1 is the more severe form with an early onset and death before ten years of age, such as the two sibs first described by Durand et al. (1966). Type 2 has a later onset, progresses at a slower rate and is characterised by the presence of angiokeratoma and survival to the second or third decade, such as the patient described by Patel et al. (1972). The similarity of phenotype between sib pairs described at that time led to the conclusion that the clinical differences between the two types is genetically determined (Kousseff et al., 1976.).

In the recent review of 77 fucosidosis patients , Willems et al. (1991) fail to delineate two clinical types, however, but describe a broad clinical spectrum between two extremes. Angiokeratoma, one of the features distinguishing type 2, may only develop at a later age, so it may only be seen in those patients who survive longer. The slower course of degeneration appears to be more common. In the survey of 77 patients (Willems 1991), 9% of patients died before the age of 5 years, whereas 64% reached the the second decade. 41% of patients died after the age of 20 years. Patients with early symptoms show a faster neurological deterioration and earlier death (82% of patients with initial symptoms appearing before 1 year,

died before reaching ten years, whereas 72% with initial symptoms after 1 year survived for more than 10 years.).

It has been suggested that the heterogeneity of phenotype observed between fucosidosis patients is a result of different mutations in the FUCA1 gene (Kousseff et al., 1976). However, interfamilial clinical variability has been found. Willems et al.(1991) compared a subgroup of patients who died before 10 years of age with a subgroup who died after 10 years of age. 11 sib pairs were concordant with division between subgroups, whereas 3 sib pairs were discordant, with one sib dying before 10 years and one after. Interfamilial clinical heterogeneity has also been reported by Romeo et al. (1977) and Durand et al. (1976).

Christomanou and Beyer (1983) described 2 Austrian sisters; one with an early age of presentation, rapid progression and death at 5 years (type 1) while her sister had a milder form with later presentation and survival after 7 years (type 2). If the clinical difference between these two patients were due to different mutations in the FUCA1 gene the parents would be compound heterozygotes and would also be clinically affected.

Two fucosidosis families were described by Willems et al. (1988a) each with type 1 and type 2 patient(s). The three patients of the first family probably had the same mutation as they had a common ancestor. In the second family, the parents of the two affected sibs were both heterozygous for the same mutation - a mutation in the last exon which obliterates an EcoR1 site (see 1.4d). This implies that the clinical heterogeneity observed in these families is not a result of different coallelic mutations in the FUCA1 gene. The difference in phenotype may be a result of environmental factors or modifying genes. No correlation between clinical type and blood group genotype, proposed as a factor in determining phenotype of fucosidosis patients, was found by Romeo et al. (1977).

1.3b. ii). Pathology.

Ultrastructural studies of biopsies and autopsies of fucosidosis patients reveal extensive vacuolation in fibroblasts, conjunctiva, peripheral nerves, liver, brain, pancreas and

skin (Loeb et al., 1969, Freitag et al., 1971; Kousseff et al., 1976, Troost et al., 1977, and Kornfeld et al., 1977). The vacuoles are limited by a single membrane and their contents are variable. Clear vacuoles and dark inclusions with dense granuolar material are found. The dark inclusions are less numerous and are found especially in the brain. This combination of dark and clear vacuoles is characteristic of fucosidosis. Inclusion bodies with alternating dark and light lamellae are also found.

1.3b. iii). Diagnosis.

Patients with severe progressive neurological deterioration and angiokeratoma corporis diffusum should be suspected of having fucosidosis, which must be confirmed or ruled out by assay of fucosidase. Thin layer chromatography (TLC) of urinary oligosaccharides can be used as a rapid screening technique for the detection of fucosidosis patients (Humbel and Collart, 1975). Affected individuals excrete fucose-containing oligosaccharide and fucoglycolipid storage products which produce a characteristic pattern on TLC plates. Diagnosis should also be confirmed by enzyme assay . The enzyme activity can be assayed in leukocytes, lymphoblasts or tissue obtained by autopsy or biopsy. Cultured fibroblasts and, especially, plasma are less reliable because of the low activity polymorphism. Only activity below 5% of controls should be taken as a definitive diagnosis of fucosidosis (Willems et al., 1991).

Prenatal diagnosis has been carried out following amniocentesis (Poenaru et al., 1976, Durand et al., 1979) and more recently by chorionic villus sampling (Young et al., 1990). Matsuda et al., (1975) prenatally misdiagnosed affected twin foetuses as being heterozygotes on the basis of enzyme activity in amniotic fluid (10% of controls) and cultivated amniotic fluid cells (30% of controls). DNA analysis using the Pvu11 and Bgl1 restriction fragment length polymorphisms or detection of the mutation by the polymerase chain reaction and restriction enzyme digestion or Southern blotting in cases where the mutation is known, would be reliable methods for the detection of affected patients and carriers. They are not, as yet, used routinely.

1.3b. iv). Atypical patients.

A few variant cases have been reported with fucosidase activities much higher than the mean activity for fucosidosis patients reported by Willems et al., (1991) (Troost et al.,1976; Blitzer et al., 1985; Sewell et al., 1987; Portoian Shuhaiber et al., 1987;). The atypical clinical features and lack of oligosaccharide secretion in urine of many of these patients suggest that at least some of these patients may have been misdiagnosed. In some cases the patient may have been homozygous for the low activity polymorphism and be affected with another disease. This was so for the patient reported by Sewell et al., (1987), who had serum and fibroblast α–L-fucosidase activity 10% of controls and lacked urinary oligosaccharide secretion. The patient was later diagnosed as having pseudohypoparothyroidism. In contrast a patient with atypical clinical features and α-L-fucosidase activity in leukocytes 31% of controls was reported by Troost et al., (1976). The low leukocyte activity could not be explained by the low activity polymorphism. Arylsulphatase A activity in this patient was also low (25% of controls).

In some cases the low fucosidase activity may be a secondary effect of an infection. Blitzer et al., 1985 reported a patient with abnormal urinary oligosaccharide secretion and low fucosidase levels . The patient was subsequently found to have AIDS (Willems et al., 1991).

Gordon et al., (1984) reported a patient with uncharacteristic phenotype and fucosidase activity assayed with 4MU of 18%, 28%, and 74% of the control mean in serum, leukocytes and fibroblasts respectively. Fucosylated oligosaccharidurie was demonstrated but glycopeptides typical of fucosidosis were not seen. Ultrastructural changes characteristic of fucosidosis patients were present in leukocytes. Activity of residual enzyme towards the substrate Fuc-α1,6-GlcNAC-Asn was similar to controls, whereas activity towards Fuc-α1,2-Gal-Glc was almost as low as that found for a typical fucosidosis patient. The authors suggest a selective defect in hydrolysis of Fuc α1-2 Gal linkages.

Alhadeff et al., (1978a) described a patient with no detectable α–L-fucosidase activity in liver towards the 4MU substrate, but with 25-30% activity towards the PNP

substate. The liver α-L-fucosidase activity towards the PNP substrate had a normal thermostability and value of Km. No activity was found in spleen towards either substrate. The accumulation of massive amounts of polar glycolipids was found in liver and smaller amounts in spleen. Partially purified liver α-L-fucosidase from the patient possessed activity towards both PNP and 4MU substrates, suggesting that material in the crude liver extract of the patient may inhibit activity towards the 4MU substrate. An extract of liver from the patient was found to inhibit activity of normal α-L-fucosidase to 4MU but not PNP, supporting this conclusion.

1.3b. v). Treatment.

At present there is no effective treatment for fucosidosis. Liver engraftment in two affected sibs did not result in stabilisation or improvement of the condition (Willems et al., 1991). Results of bone marrow transplantation in fucosidosis dogs have been encouraging however (Taylor et al., 1988; see section 1.5).

Enzyme replacement therapy has given positive results in some other lysosomal storage diseases. Clinical improvement was observed in Gaucher patients following intravenous infusion of glucocerebrosidase. The enzyme was targetted to macrophages by modification of the glycosylation of the native enzyme to expose mannose residues which would be bound by mannose specific receptors on macrophages (Barton et al., 1990, Barton et al., 1991). Galaptin has been used as a transport vehicle for delivery of α-L-fucosidase to fucosidase deficient cells in vitro (Allen et al., 1990). Galaptin-fucosidase conjugates may therefore be useful for enzyme replacement of fucosidosis in cells bearing galaptin receptors.

Fucosidosis is a good candidate for somatic gene therapy in the future. It is a single gene defect, is recessive in nature and appears to be almost ubiquitously expressed, not requiring a high degree of regulation of expression. The enzyme would probably be therapeutic at low levels (individuals that are homozygous for the asymptomatic low activity polymorphism have plasma fucosidase activity approximately 30% of controls). The severity

of the disease and lack of alternative conventional therapies also make it a good candidate. However, gene therapy will still face the same barriers to treatment of neurologicl storage disorders as allogeneic BMT -namely the delivery of the enzyme across the blood brain barrier to the CNS. Substantial levels of enzyme replacement in neural tissues (over 20% of normal activity) and histological improvement have been shown in fucosidosis dogs 6 months after bone marrow engraftment (Taylor et al., 1989). A full length fucosidase cDNA clone has been introduced into retroviral expression vectors and used to infect fibroblasts from a fucosidosis patient. Infected cells expressed fucosidase at levels approximately 80 times those found in normal human fibroblasts (Anson et al., 1990). High titre virus will be used to infect canine bone marrow cells, with the aim of attempting gene replacement therapy by transplantation of autologous infected bone marrow cells in fucosidosis dogs. Therapy will have to be performed before the onset of clinical symptoms if disease progression is to be prevented.

1.3c. Nature of the enzymic defect.

1.3c. i). Residual activity.

Fucosidosis patients typically have negligible activity in all tissues. The mean α–L-fucosidase activity in fibroblasts (43 patients), leukocytes (47 patients), lymphoblastoid cell lines (4 patients), plasma (19 patients), and liver (14 patients) were 1%, 1%, 0%, 4%, and 0%, of the control mean respectively (Willems et al., 1991). No correlation has been found between the level of residual activity and the clinical severity. The mean residual leukocyte activity of patients who died before 10 years of age (7 patients) was not significantly different (>0.5) from that of 21 patients alive after 10 years.

Activities of certain other lysosomal enzymes, such as α-D-mannosidase are significantly increased above control levels in tissues of fucosidosis patients (Matsuda et al., 1973).

1.3c. ii). Cross reacting immunological material (CRIM).

Anti fucosidase CRIM was measured in fibroblasts and lymphoblastoid cell lines using

a quantitative ELISA assay with antibodies raised in rabbits against α-L-fucosidase (DiCioccio et al., 1986; Willems et al., 1988b). The mean anti-fucosidase CRIM was 2.2 + 1.3% and 1.3 + 0.9% of the control mean in fibroblasts (21 patients) and lymphoblasts (3 patients) respectively.

Obligate heterozygotes had CRIM levels of 33.7 + 24% and 50.9 + 43.4% of the control mean in fibroblasts and lymphoblasts respectively.

1.3c. iii). The residual protein.

Biochemical studies on the residual α–L-fucosidase protein have been hampered by the very low amount present. Alhadeff and Andrews-Smith (1980b) partially purified the α-L- fucosidase protein from the liver of a patient with no detectable activity using aminohexanoylfucosamine and concanavalin A affinity chromatography. A 4-5 fold increase in the value of Km (700-750uM) using 4MU as substrate and a decrease in thermostability at 55^{o}C was found for the residual α-L-fucosidase compared to the normal liver enzyme. The activity pH-curve of the mutant enzyme was very similar to that of the control enzyme at pH 5-8, but differed at pH 3-4.5; the normal enzyme having 50% maximal activity at this pH whereas the mutant enzyme had no activity. Isoelectric focusing of the mutant liver enzyme revealed a single major peak with a pI of 5.8 and some minor forms (Alhadeff and Andrews-Smith, 1980b). A ten fold increase in Km was also found for the leukocyte enzyme from one patient by Troost et al., (1976). The residual fucosidase from the sera of two fucosidosis patients described by DiMatteo et al., (1976) showed a single major peak following isoelectric focusing and a decrease in thermostability compared to the normal enzyme.

1.3c. iv). Post translational processing.

DiCioccio et al., (1989) found that the percentage of α–L-fucosidase protein released extracellularly was higher in lymphoblastoid cell lines of four fucosidosis patients than of controls. 64-85 % of total α-L-fucosidase protein was released from cell lines from four patients, whereas only 35% was released from 19 controls. The rate of secretion was also

considerably increased.

Abnormal expression of α-L-fucosidase was found in lymphoblastoid cell lines of four patients using pulse chase experiments (DiCioccio et al., 1989). In control cells a precursor of 58,000 Da is processed to a 60,000 Da intracellular form and a 62,000 Da extracellular form. In two unrelated patients the intracellular 58,000 Da form and the 62,000 Da extracellular form were seen but the 60,000 Da intracellular form was absent. In cells from two fucosidosis siblings a 56,000 Da intracellular form was processed to a 60,000 Da extracellular form. All four patients had a carbohydrate free polypeptide, following deglycosylation, of the same molecular weight as controls (52,000 Da). Enzyme from the two affected sibs was therefore less glycosylated than controls and the two unrelated patients. All four patients therefore had decreased amounts of catalytically inactive enzyme which was hypersecreted.

Johnson and Dawson, (1985) investigated the quantity and processing of α–L-fucosidase in fibroblasts of 11 patients. In 8 of these fibroblast cell lines a virtual absence of CRIM was reported, (however one was later described by Dioccio (1989) as having small amounts of fucosidase which was hypersecreted). Two of the 11 patients had normal amounts of the 53,000 Da form but a virtual absence of the 50,000 Da form. One had a low level of both forms.

1.3d. Storage products.

The deficiency of α-L-fucosidase results in the accumulation of the fucoglycoconjugate substrates of the enzyme in lysosomes. Storage products have been found in all tissues investigated including liver, spleen, brain, fibroblasts, pancreas, lung kidney, lymph node; and are excreted in the urine (Tsay and Dawson, 1975; Tsay et al., 1976). The diversity of fucose-containing storage products found in fucosidosis patients reflects the wide range of fucoglycoconjugates in the cell.

A large proportion of the storage products are glycoasparagines (Yamashita et al.,

1979). This large proportion of glycopeptides is unique to fucosidosis among the glycoproteinoses. All the glycopeptide storage products have a Fuc 1-6 GlcNAc-Asn group at the reducing end. Fuc 1-6 GlcNAc-Asn is also one of the major storage products. Accumulation of these products suggests that cleavage of the GlcNAc-Asn linkage by aspartylglucosaminidase is inhibited by the steric hindrance of the fucose attached to the GlcNAc group. This has been confirmed by studies in vitro by Tachibana et al., (1981). Some oligosaccharides also accumulate in fucosidosis (Nishigaki et al., 1978). These all have Man-GlcNAc at the reducing end which suggests that they arise from glycoasparagines which lack a fucose on GlcNAc residue attached to asparagine and are susceptible to hydrolysis by aspartylglucosaminidase . Storage of glycolipids has also been reported, including the H antigen glycolipid (Dawson and Spranger, 1971).

Many of the storage products contain other sugars such as mannose and galactose at the non-reducing terminal in spite of the presence of other lysosomal enzymes such as α-mannosidase and β-galactosidase. This suggests that cleavage of fucose from the glycoconjugates is a prerequisite for the action of other lysosomal enzymes on the substrate, and that it is therefore one of the initial steps in glycoconjugate breakdown.

Ng Ying Kin (1987) reported that different patterns of storage products were secreted in the urine of type 1 and type 2 patients. Fuc1-6GlcNAc -Asn excretion was significantly higher for type 2 patients than for type 1 and the product Gal 1-4(Fuc 1-3) GlcNAc 1-2Man 1-3/6Man 1-4GlcNAc was present in greater quantities in urine from a type 1 than a type 2 patient. The authors suggested that the different patterns reflect different substrate specificities of the residual α-L-fucosidase in the two forms of the disease, type 1 having a deficiency of Fuc 1-3 Glc specific activity, and type 2 patients having both 1,3 and 1,6 fucosidase activities deficient. However, in all other reported cases all types of α-fucose linkages have been found.

Abnormal expression of Lewis blood group substances in fucosidosis has been reported. Most fucosidosis patients are Lewis a+ b+ which is uncommon in the normal population. Patients whose parents were a- were found to be a+ and patients of Lewis group

b+ had parents of b-. This probably results from the accumulation of Lewis group substances in fucosidosis (Staal et al., 1977). Contrary to earlier reports, no correlation was found between the Lewis blood group genotype and clinical severity (Romeo et al., 1977).

Accumulation of saposins A and D has been found in the liver of patients with fucosidosis, approximately 70 and 20 fold higher than control levels respectively (Morimoto et al., 1990). Neither saposins A or C stimulate α-L-fucosidase activity, using 4MU as substrate, but it is unknown if this is also the case for glycolipid substrates. Little fucoglycolipid is stored in the brain of fucosidosis patients, and a lower level of saposin accumulation in the brain was found. The authors propose that this parallel storage of saposin and glycolipid may indicate a complex between the two.

1.4. Molecular genetics.

1.4a). Chromosomal Location.

FUCA1 was origionally assigned to chromosome 1 by assaying fucosidase activity in somatic cell hybrids, and linkage of the isoenzyme polymorphism to the Rhesus blood group (Corney et al., 1977). Southern blot analysis of somatic cell hybrid cell lines using α-L-fucosidase cDNA probes also mapped the gene to chromosome 1. In situ hybridization further localised the gene to 1 p36.1 - p34.1 (Fowler et al., 1986).

1.4b).The FUCA1 structural gene.

1.4b i). The cDNA.

α-L- fucosidase cDNA was initially cloned by screening a λ gt11 human liver cDNA library with rabbit anti human α-L-fucosidase antibodies (de Wet et al., 1984; Fukushima et al., 1985). A cDNA clone representing 80 % of the coding region was isolated and sequenced. The frequency of fucosidase sequences in the library was 0.0018% (Fukushima et al., 1985).

Occhiodoro et al., (1989) subsequently cloned and sequenced the remaining cDNA. The complete cDNA is 2053 base pairs (bp) long and has an open reading frame of 1383 bp

coding for 461 amino acids. A consensus polyadenylation signal AATAAA is found 28 bp upstream from the poly(A) tail. The cDNA codes for a mature protein of 439 amino acids, and a hydrophobic 5' signal sequence of 22 amino acids. Four potential glycosylation sites have been identified (Fukushima et al., 1985).

The identity of the cDNA has been confirmed by colinearity of fucosidase amino acid sequence and the nucleotide sequence (Fukushima et al., 1985), chromosomal assignment to 1p 34.1-36.1, and detection of mutations causing fucosidosis within the cDNA.

1.4b. ii). The FUCA1 genomic structure.

Southern blot analysis and restriction endonuclease mapping, using α-L-fucosidase cDNA probes suggest that the gene is approximately 22 kb with at least 7 exons (Darby et al., 1988).

1.4b. iii). The mutant α-L-fucosidase gene.

The LOD score between fucosidosis and the FUCA1 RFLP markers was shown to be significant (Darby et al., 1988). Linkage has also been shown between Fu1/Fu2 protein polymorphism and fucosidosis. This provides evidence that the fucosidosis-causing mutation(s) lie in the fucosidase structural gene, FUCA1.

1.4c. Restriction fragment length polymophisms

Two restriction fragment length polymorphisms (RFLP's) have been identified at the FUCA1 locus (Darby et al., 1988) each with two codominant alleles in Hardy Weinberg equilibrium. Pvu11 identifies a 2 allele polymorphism with bands at 7.0kb (p1) or 6.0kb (p2), with a frequency in the Caucasian population (n=122) of 0.7 (p1) and 0.3 (p2). A two allele polymorphism is also identified using Bgl1 with bands of 12 kb (p1) or 6.5 and 5.5 kb (p2) and a frequency of 0.63 (p1) and 0.37 (p2) in 46 unrelated Caucasians. Both RFLP's are in strong linkage disequilibrium with each other, reflecting the fact that they are less than 1kb apart. Haplotypes for Pvu11 and Bgl1 in FucA1 were constructed for a control group of

45 US and 46 Belgium Caucassians (Willems et al., in press). The US control group showed almost complete linkage disequilibrium between the 7 kb Pvu11 and 12 kb Bgl1 allele (haplotype 1-1) and between the 6kb Pvu11 and the 6.5 and 5.5 kb Bgl 1 allele (haplotype 2-2). The standard linkage disequilibrium coefficient (r) was 0.95. Significantly ($P < 0.001$) lower linkage disequilibrium is found in the Belgium Caucassian group (r=0.53).

The Pvu11 and Bgl1 polymorphisms have polymorphism information content (PIC) values of 0.33 and 0.36 respectively, and a combined PIC value of 0.38. They could therefore be useful in prenatal diagnosis.

Linkage disequilibrium has been detected between the Pvu11 RFLP and the α -L-fucosidase isoenzyme polymorphism ($p = 0.008$ with the Fisher exact test). The Pvu11 7 kb allele is associated with Fu1, and the Pvu11 6kb allele is associated with Fu2.

No polymorphisms were detected using the restriction enzymes Ava 2, BamH1, Bgl 2, EcoR1, EcoR5, Hinc 2, Hind 3, Kpn 1, Mbo 1, Msp 1, Pst 1, Rsa 1, Sac 1, Sst 1, Stu 1, Taq 1, and Xmn 1 in 8 normal unrelated Caucasians, using fucosidase cDNA probes (Darby et al., 1986;1988).

1.4c. i). Haplotypes of fucosidosis patients.

The standardised linkage disequilibrium coefficient (r) was 1.0 between the 7kb Pvu11 and the 12kb Bgl1 allele (haplotype 1-1) and between the 6kb Pvu11 and the 6.5/5.5kb Bgl1 allele (haplotype 2-2) for a group of 17 patients (Willems et al., in press).

1.4d. Mutations in the fucosidase gene.

i). The EcoR1 mutation.

Southern blot analysis of DNA from a subset of fucosidosis patients first identified a mutation which results in obliteration of an EcoR1 site in the open reading frame of the FUCA1 gene (Willems et al., 1988). Kretz et al., (1989) subsequently used PCR to amplify

the region of the gene containing the altered restriction site using primers complementary to the cDNA sequence. Sequence analysis revealed a C - T transition at the last position of the EcoR1 recognition sequence, which results in generation of a stop codon 120bp upstream from the normal stop codon, i.e. GAATTC - GAATTT. The mutation is in the last exon of the fucosidase gene, at position 1053 of the cDNA sequence. Patients with this mutation have very low fucosidase CRIM (0.5 - 2.2 % of controls) and negligible α-L-fucosidase activity (0.02 - 0.05 % of controls), (Willems et al., 1988). The mutation would presumably result in production of a truncated polypeptide of molecular mass 5000 lower than the wild type. This was not found in lymphoid cells from two siblings who were heterozygous for this mutation, however (DiCioccio and Brown, 1988). The premature stop codon may result in production of unstable mRNA or a mutant protein that is unstable.

The EcoR1 mutation has been found in seven fucosidosis patients. Three unrelated patients and one sibling pair were homozygous for the mutation and another sibling pair were compound heterozygotes for the EcoR1 mutation and a different mutation. The five patients homozygous for the EcoR1 mutation have a variety of phenotypes, ranging from rapid neurological decline and death at five years in one patient, i.e. type 1, to a slow decline and survival to over 20 years in another, i.e. type 2.

So far all the alleles in which the EcoR1 mutation has been identified have had the haplotype 2 - 2 and none has a de novo mutation. Consequently, Willems et al., (in press) suggest that the mutation has occurred only once, in a 2 - 2 individual, and that all patients with this mutation have a common ancestor, although they are of different ethnic origins (2 Italian families, 2 French families and one Cuban).

ii). The deletion mutation.

A deletion mutation has been identified in two Algerian siblings by Southern blotting analysis. At least the last two exons at the 3' end of the the FUCA1 gene are deleted. Both siblings had haplotype 1 - 1. Any mutant protein encoded by this truncated gene would be at least 6kb shorter than the normal enzyme protein. Enzyme activity and CRIM were negligible

in both patients (Willems et al., in press).

iii). Other mutations.

Southern blot analysis with several different restriction enzymes of DNA from fifteen other fucosidosis families did not reveal any major gene abnormalities (Willems et al., 1988). Fucosidosis in these families therefore probably results from small deletions, insertions or point mutations.

1.4e. Messenger RNA.

i). Controls.

Northern blotting of poly A^+ mRNA and hybridization to fucosidase cDNA probes has identified an α-L-fucosidase mRNA of 2.3 kb (Guazzi et al., 1989; O'Brien et al., 1987). This is in accordance with the subunit size of the protein. Guazzi et al. also identified additional bands of 8 and 12 kb, possibly representing unprocessed or partially processed fucosidase mRNA.

ii). mRNA production in fucosidosis patients.

Guazzi et al., (1989) investigated the production of fucosidase mRNA in six unrelated fucosidosis patients. Poly A^+ RNA was isolated and analysed by Northern blotting using fucosidase cDNA probes. In two patients mRNA was not detected, whereas in three patients mRNA was normal in both size and amount. No abnormalities in the DNA were observed by Southern blotting in any of these patients. The sixth patient produced a greatly decreased amount (approximately 30 %) of mRNA of the normal size. This patient was homozygous for the EcoR1 mutation. These studies suggest that at least two additional mutations exist in fucosidosis, one with normal mRNA production and one without.

Fucosidase mRNA was absent from fibroblasts of 4 Mexican fucosidosis patients, as detected by Northern blotting (O'Brien et al., 1987).

1.4f. The FUCA1L pseudogene.

FucA 1 cDNA probes also hybridize to a second site of homology on chromosome 2 (Fowler et al., 1986). This site has been cloned and restriction mapping has shown that the genomic clone is co-linear with at least 70% of the fucosidase cDNA (Carritt and Welch, 1987). The absence of introns suggests that the FUCA1L locus represents a processed pseudogene. A two allele Bcl 1 RFLP has been mapped to this locus, which is detected by FucA 1 cDNA probes as well as the Fuc1L genomic clone. Bands of 12 kb or 8.1 and 3.9 kb are seen on Southern blots of Bcl1/Pst1 digested DNA, with frequencies of 0.58 and 0.42 respectively (Willems et al., 1990).

1.5. Canine fucosidosis.

A progressive neurovisceral storage disorder in English Springer spaniels resulting from a severe deficiency of α–L-fucosidase was first reported by Hartley et al.,(1982) in Australia and by Littlewood et al.,(1983) in the U.K. Symptoms first appear at one to two years of age . Progressive ataxia, tremor, inability to swallow, blindness and deafness follow and death occurs between 3 and 5 years, usually by euthanasia. Vacuolation of intra and extraneuronal tissues is found, as in the human disease (Kelly et al., 1983). Canine fucosidosis is comparable to the milder human phenotype with adult onset of symptoms.

Residual α-L-fucosidase activity is less than 5% of the control mean (Abraham et al., 1984) and the canine equivalent of both human fucosidase types 1 and 2 are deficient. Cross reacting immunological material was shown to be absent from different tissues of fucosidosis dogs by immunodiffusion or Western blotting, using antibodies raised against normal canine liver α-L-fucosidase, (Barker et al., 1988a). As in human fucosidosis fucoglyconjugates accumulate in the tissues and are secreted in the urine as a result of the enzymic deficiency. The three main storage products in the brain of an affected dog were shown to be:

1. Fuc-GlcNAc-Asn

2. Gal-GlcNAc-Man-Man-GlcNAc-GlcNAc-Asn

I I

Fuc Fuc

3. Fuc

I

Gal-GlcNAc-Man

\

Man-GlcNAc-GlcNAc-Asn

/ I

Gal-GlcNAc-Man Fuc

I

Fuc

(Abraham et al., 1984). These glycoasparagines also accumulate in the human disease. Differences have been found between the structures and patterns of accumulation of storage products between different tissues of a dog affected with fucosidosis (Barker et al., 1986). Species-specific variability in the structure of stored metabolites and in the particular cells affected has been found between canine and human fucosidosis (Alroy et al., 1985).

The canine α-L-fucosidase gene has not been characterised yet. Barker et al., (1988b) demonstrated by Southern blot analysis of DNA from affected dogs using the human fucosidase cDNA probes, that no gross gene deletions insertions, or rearrangement occurs in canine fucosidosis.

Pedigree studies have shown that canine fucosidosis is inherited in an autosomal recessive manner. A screening programme for heterozygotes based on measurement of plasma and leukocyte α-L-fucosidase has been undertaken with the aim of eradicating this disease

from the breeding stock. The incidence of the fucosidosis gene in English Springer spaniels is approximately 0.13 (Barker et al., 1988b). This may be high because of non-random mating and the biased submission of animals for testing.

Canine and human fucosidosis both result from a severe deficiency of the same enzyme leading to accumulation of similar storage products and development of a similar phenotype. Canine fucosidosis can therefore be used as a model for the human disease. Affected English Springer spaniels have been used in studies to determine the efficacy of bone marrow transplantation for the treatment of fucosidosis. Bone marrow transplantation carries the serious risk of the development of graft verses host disease and graft rejection with resultant aplasia. It is therefore preferable to evaluate the effects of these treatments on animal models prior to treatment of affected children. Canine fucosidosis provides such a model. α-L-fucosidase levels in plasma leukocyte and tissues of fucosidosis dogs, increased significantly following total lymphoid irradiation and allogeneic bone marrow transplantation (Taylor et al., 1988). Substantial enzyme replacement (over 20% normal activity) has also been observed in neural tissues behind the blood brain barrier. A decrease in the severity of histological abnormalities was also observed. (Taylor et al., 1989). Canine fucosidosis will also be useful in assessing the effectiveness of gene therapy (Anson et al., 1990).

Aims.

The aim of the project is to characterise the defect underlying fucosidosis at the protein, RNA and DNA level in six fucosidosis patients. Identification of mutation(s) causing fucosidosis would aid diagnosis and may allow correlations between genotype and phenotype to be made which would help prognosis and genetic counselling. A further aim of the project is to investigate the molecular basis of canine fucosidosis in English Springer spaniels, an animal model for the human disease.

Materials and methods.

The reagents used are listed in Appendix 2. Polyclonal and monoclonal anti-fucosidase antibodies were provided by Dr.J. Alhadeff. Fucosidase cDNA probes were provided by Dr. J. S. O'Brien. Primers 4 and 5 were provided by Dr. P. Willems.

2.1. Tissue culture

Growth medium:

Hams medium (Gibco Ltd); 8% (v/v) fetal calf serum; 2% (v/v) newborn fetal calf serum; 2mM glutamine; 60µg/ml benzyl penicillin, 167 µg/ml streptomycin sulphate.

Trypsin dilution buffer:

6g/l Sodium chloride, 3g/l Sodium citrate, pH 7.8, autoclaved.

2.1a. Culture of fibroblast cell lines.

Fibroblasts were grown in sterile 75cm^2 tissue culture flasks in 20ml of growth medium which was changed twice a week. The flasks were incubated at 37^{o}C in a 5% CO_2 incubator. The fibroblasts were subcultured or harvested when they had reached confluence. The passage number of the fibroblasts cell lines did not exceed 15. All procedures were carried out aseptically.

2.1b. Subculturing and harvesting of fibroblasts.

The growth medium was removed from the cells and the fibroblasts were washed with trypsin dilution buffer. The cells were then covered in a solution of 0.25% trypsin (v/v) in the same buffer and incubated for 5 min at 37^{o}C. The flask was agitated to detach the cells. The cell suspension was then transferred to a sterile tube containing 1 ml of growth medium and was either spun for 5 min at 600g to collect cells for storage, or distributed among three sterile flasks with more growth medium for further growth.

2.1c. Storage of fibroblasts.

PBS:

8g/l NaCl, 0.2g/l KCl, 1.44g/l Na_2HPO_4, 0.24g/l KH_2PO_4, pH7.4, autoclaved.

DMSO medium:

7.5% (v/v) dimethyl sulphoxide in Hams medium, filter sterilised.

i). For subsequent enzyme assay or DNA extraction.

Following harvesting, the fibroblast pellet was resuspended in sterile PBS and spun once more. The cells were resuspended in 50 µl of sterile water and rapidly frozen in a dry ice-methanol bath. They were then stored at -20°C.

ii). For subsequent reculture.

The fibroblast pellet was resuspended in DMSO medium and left at 4°C for 18 hours. The suspension was then frozen in liquid nitrogen vapour at approximately 1°C per min until it reached -70°C. The fibroblasts were stored in liquid nitrogen at -70°C.

2.1d. Transformation of lymphocytes with Epstein Barr Virus.

Growth medium for transformed lymphocytes:

RPMI 1640 (containing $NaHCO_3$) 10% (v/v) fetal calf serum (FCS), 50 µg/ml gentamycin, 1µg/ml cyclosporin.

5ml of fresh blood (collected in heparin) was diluted with RPMI 1640 medium (Gibco) with 5% fetal calf serum (FCS) to 10ml. The diluted blood was layered onto 5ml of Ficoll and spun at 2000g for 20min. The layer of leukocytes was extracted and washed with RPMI+5%FCS and then spun at 600g for 7min. Each leukocyte pellet was incubated with approximately 20-40 x 10^6 Epstein Barr virus particles (EBV, strain b958) at 37°C for 2hrs in 5% CO_2.

The transformed cells were washed in RPMI+5%FCS and spun at 600g for 7min. The cell pellet was resuspended in 6ml growth medium. Approximately 10^6 cells were added to each well of a sterile culture plate. The outside wells were filled with sterile water to provide a humid atmosphere. The cells were incubated at 37°C in 5%CO_2 for approximately 14 days after which they were transferred to sterile tissue culture flasks.

2.1e. Culture of lymphoblast cell lines.

The lymphoblasts were grown in growth medium in sterile 75cm^2 culture flasks. The medium was replaced approximately twice a week. The lymphoblasts were harvested by aspiration with a pipette. They were stored as for fibroblasts.

2.2 Enzyme studies.

2.2a. Preparation of leukocytes and plasma for assay of enzymes.

The sample of heparinised blood (10 ml) was spun for 10 min at 2000g and the plasma removed and stored frozen for later use. The layer of leukocytes was transferred to a separate tube to which 1 ml of NaCl (0.9% w/v) was added. 0.9% NaCl was also mixed with the remaining red cells which were spun again at 2000g. The second layer of leukocytes was combined with the first.

The leukocytes were spun for 5 min at 600g and the supernatant discarded. The saline wash was repeated and 1.5 ml of cold water was added to the leukocyte pellet to lyse any remaining red cells. The pellet was resuspended with a pasteur pipette and after 90 seconds 0.5 ml of cold 3.6% (w/v) NaCl was added and mixed. The suspension of leukocytes was spun at 2000g for 10 seconds and the supernatant removed. The water wash was repeated and the leukocyte pellet was then resuspended in 100µl of cold water. The resuspended leukocytes were rapidly frozen in a dry ice-methanol bath and stored at -20°C until required. A short time prior to the enzyme assay, the leukocyte pellets were thawed and sonicated for 10 seconds at amplitude 6 in an MSE Soniprep ultrasonicator.

2.2b. Preparation of fibroblasts for assay of enzyme.

The fibroblast cell pellets were thawed and sonicated for 10 seconds, at amplitude 6 in an MSE soniprep ultrasonicator. The lysate was spun in a microfuge for 3 min and the pellet of cell debris discarded.

2.2c. Assay of α-L-fucosidase.

5μl of supernatant of extracts of fibroblasts or leukocytes or 5μl of plasma was assayed using 0.64mM 4-methyl umbelliferyl-α-L-fucoside in 0.4M sodium acetate buffer in an incubation mixture of 200μl at 37°C for 30 min. The reaction was stopped by the addition of 2.3ml of glycine/NaOH, pH10.4. The fluorescence was measured in a Perkin Elmer spectrofluorimeter with an excitation wavelength of 360 nm and emission wavelength of 440 nm. If the reading was too high, the cell extract was diluted and the assay repeated.

All assays were performed in duplicate. A control of buffered substrate and water, with no cell extract, was always included (which gives the fluorescence of unconverted substrate). The value of the substrate blank was subtracted from all the other readings. The fluorescence of a standard of 1 nanomole of 4 methylumbelliferone was measured for each set of assays and the fluorescence of the tests related to this value.

The activity was expressed as nanomoles of 4 methylumbelliferone released per milligram of total protein (or ml of plasma) per hour.

2.2d. Assay of α-D-mannosidase activity.

α–D-mannosidase activity was assayed as for α-L-fucosidase, except that McIlvaine buffer (0.2M sodium phosphate adjusted to pH 4 with 0.1M sodium citrate) was used as buffer, and 4 methylumbelliferyl-α–mannoside was used as substrate at 1.69 mg/ml. The reaction was incubated at 37°C for 30 min.

2.2e. Total β-hexosaminidase activity assay.

Hexosaminidase activity was assayed in McIlvaine buffer at pH 4.5 using 4 methylumbelliferyl-2-acetamido-2-deoxy-β D pyranoside (1.1 mg/ml) as substrate. The assay mixture of 200μl was incubated at 37°C for 10 min.

2.2f. Protein determination.

The total protein concentration of fibroblast or leukocyte lysates was determined according to the method of Bradford et al. (1976). Solutions of 500μl of cell extract containing between 5 and 25 μg of protein were prepared and added to an equal volume of Pierce Protein Assay Reagent. The tubes were inverted to mix the contents and after 5 min the absorbance at 595 nm was read against a water blank. A standard curve of absorbance against protein concentration was constructed using standard solutions of BSA of concentrations between 5 and 25 μg/ml. The concentrations of the unknown samples were read from the standard curve. All assays were performed in duplicate. The absorbance of a reagent control containing no protein was subtracted from all the readings.

2.2g. Investigation of the pH-dependence of α-L-fucosidase activity.

The α-L-fucosidase activity of fibroblast extracts was assayed using 4 methylumbelliferone-α-L-fucoside as substrate in sodium acetate buffer at a series of pH values between 3.0 and 7.5 with increments of 0.5 pH units. The assays were carried out as in 2.2c. Each assay was performed in duplicate and the fluorescence reading of a substrate blank at each pH was subtracted from the readings at each pH.

2.2h. Investigation of the effect of substrate concentration on α-L-fucosidase.

A series of solutions of the substrate 4 methylumbelliferyl-α–L-fucoside at different concentrations was made in 0.4 M sodium acetate buffer at pH 4.5. The α-L-fucosidase

activity of fibroblast extracts was assayed at each substrate concentration and the value of Km calculated from a Lineweaver-Burk plot, using a line of best fit. The range of concentrations was from 0.1mM to 1.0mM, covering the approximate expected value of Km, as determined in a preliminary experiment and from previously published work.

2.2i. Thermostability of α-L-fucosidase

Samples of fibroblast extracts were preincubated for 30 min at $0^{o}C$, $24^{o}C$, $30^{o}C$, $37^{o}C$, $50^{o}C$, or $60^{o}C$ before being assayed for α-L-fucosidase activity at pH 4.5, using the standard procedure. The activity obtained after pre-incubation at each temperature was compared (%) with the activity measured after pre-incubation at $0^{o}C$.

2.3. Thin Layer Chromatography of urinary oligosaccharides and glycopeptides.

2ml of urine was deionised with Amberlite mixed bed resin and then freeze dried. The resultant dry material was resuspended in distilled water to achieve comparable creatinine concentrations in all the samples. The treated urines samples were applied as a 1cm line to the bottom of silica gel TLC plates, using a Hamilton syringe. The samples were dried between each application. The silica gel plates were placed in tanks containing a solution of propan-1-ol, acetic acid and water (3:3:2). When the solvent had travelled approximately 10cm up the plate, it was taken out of the tank and dried, and then replaced in the tank. The solvent was allowed to reach the top of the plate in the second development. The plate was removed, dried and was then sprayed with a freshly prepared solution of orcinol (0.2g/100ml) in 5% (v/v) sulphuric acid in methanol. The sprayed plate was heated for 10 min at $100^{o}C$ to detect reducing sugars.

Fucoglycoconjugate storage products isolated from the brain of a dog affected with fucosidosis were used as standards (fig7.2). 5μg of standard was loaded each time.

2.3.a. Determination of total sugar content.

1µl, 10µl, or 20µl of untreated urine was made up to 200 µl with water, and added to 800 µl of orcinol sulphuric acid reagent (0.2 % orcinol in concentrated H_2SO_4). The solution was incubated at 80°C for 15 min and then cooled to room temperature. The absorbance of the reaction mixtures was read at 420 nm. A standard curve of absorbance against sugar concentration was constructed, using fucose solutions of different concentrations (1 µg to 20 µg). The concentration of reducing sugar in the urine samples was calculated by reference to the standard curve.

2.4. Immunological techniques.

2.4a. Immunoprecipitation of α-L-fucosidase enzyme.

Polyclonal goat anti human α-L- fucosidase was diluted serially, 100, 500, 1,000, 5,000, 10,000, 50,000 and 100,000 fold. 100µl of each diluted antiserum was incubated with an equal volume of suitably diluted fibroblast extract at 37°C for 2 hours, followed by overnight at 4°C. 100 µl of a 10% solution of *Staphylococcus Aureus* protein A was added to enhance precipitation of the antibody-antigen complex. The mixture was incubated for 30 min at 37°C and then spun in a microfuge for 4 min. 100µl of the supernatant was assayed for α–L-fucosidase activity by the standard procedure. Controls of antiserum incubated with PBS (to replace the fibroblast extract), and fibroblast extract with PBS (to replace the antiserum), were included. The equivalence point for precipitation of the α–fucosidase in various samples was obtained by plotting activity remaining in the supernatant against antiserum dilution.

2.4b. Quantitative measurement of α-fucosidase protein by double antibody sandwich ELISA technique.

Blocking solution: BSA (3% w/v), sodium azide (0.02% w/v).

50µl of a solution of polyclonal goat anti-human IgG (20µg/ml in PBS) was put in the wells of a microtitre plate. PBS was used in control wells. The plates were incubated at room

temperature for 1.5 hr. The wells were washed twice with PBS and then 250µl of block solution was added to each well. The plates were incubated at room temperature overnight and then washed twice with PBS. 50µl of extracts of sonicated fibroblasts or lymphoblasts which had been diluted to 20µg/ml, 40µg/ml, or 80µg/ml in blocking solution was next put in the wells; PBS was put in a control well. The plates were incubated for 1.5hr at room temperature and then washed four times with PBS.

50µl of monoclonal rat antihuman α-L-fucosidase IgG, diluted 1000 fold in blocking solution was then added, again using PBS as control. After incubation for 1.5hr at room temperature the wells were washed four times with PBS. 100µl of alkaline-phosphatase-conjugated goat anti-rat IgG serum, diluted 500 times in blocking solution was added to each well. The plates were incubated for 1hr and then washed four times with PBS, followed by twice with 10mM diethanolamine, pH 9.5, containing 0.5mM $MgCl_2$. 50µl of substrate solution, 1mg/ml *p*-nitrophenyl phosphate in 10mM diethanolamine, was added to each well and the plates were incubated for two hours to overnight at room temperature. The absorbance at 405nm of the released *p*- nitrophenol was measured using an ELISA plate reader.

Results were calculated by subtracting the average background absorbance with no antigen from all readings. The results were expressed as a percentage of absorbance of control fibroblasts or lymphoblasts.

2.5. DNA- and RNA-based techniques.

2.5a Preparation of DNA from blood.

TE: 10mM Tris, 1mM EDTA, pH 7.5.

Blood was stored in 10ml EDTA tubes at -20°C. 40ml of ice cold distilled water was added to 10ml of blood and mixed. The tubes were spun at 2000g for 20 mins. The pellet was resuspended in 25ml NP40 (0.1% v/v) by mixing with a whirlimixer. The solution was spun at 1000g for 20 min at 4°C. The nuclear pellet was homogenised in 7ml 6M guanidinium

hydrochloride and 0.5ml 7.5M ammonium acetate. 0.5ml of Sodium sarkosyl (20% w/v) and 150µl proteinase K (10mg/ml) were added and the solution incubated at 60°C for 90min. 17ml of absolute ethanol was added and the DNA spooled. The DNA was redissolved in TE overnight. The DNA was reprecipitated by adding one tenth volume of 3M sodium acetate and 2 volumes of ethanol. It was spooled a second time and dissolved in TE overnight.

2.5b. Preparation of cytoplasmic RNA.

PBS: 8g/l NaCl, 0.2g/l KCl, 1.44g/l Na_2HPO_4, 0.24g/l KH_2PO_4, pH 7.4.
Lysis buffer: 0.14M NaCl, 1.5mM $MgCl_2$, 10mM Tris pH 8.6.
PK buffer: 200mM Tris pH 7.5, 25mM EDTA, 300mM NaCl, 2% SDS.

i). Fibroblasts.

Only freshly harvested (not frozen) fibroblasts were used for RNA extraction. The fibroblast cell pellets were washed twice in sterile PBS before suspension in lysis buffer containing 0.5 % (v/v) NP-40 and 10mM vanadyl ribonucleosides. An equal volume of lysis buffer containing 24 % (w/v) sucrose and 1% (v/v) NP-40 and 10mM vanadyl ribonucleosides was put in a sterile siliconised Corex tube. The resuspended cell pellet in lysis buffer was carefully layered onto the sucrose solution. The tube was then spun at 10,000g for 25 min at 4°C.

Following centrifugation two layers were visible. The top layer, containing the RNA, was removed to a new sterile tube, and 2ml of PK solution containing 200µg/ml of proteinase K was added. The solution was incubated at 37°C for 30 min and then extracted with an equal volume of phenol once, phenol/chloroform twice and chloroform once. The phenol used in this step had been equilibrated with TE pH 5.0 made with Diethyl pyrocarbonate (DEPC)-treated water. 2.5 volumes of ethanol was then added and the tube left at -20°C for 18 hours. The solution was then put in another sterile siliconised Corex tube, and spun at 10,000g for

20 min. The pellet was resuspended in DEPC-treated water and the concentration of RNA estimated by absorbance at 260 nm. The RNA was stored at -20^{o}C after the addition of a tenth volume of 3M sodium acetate pH 5.5 and two volumes of ethanol.

ii). Leukocytes.

An equal volume of dextran(6% w/v) was added to whole blood in a sterile tube. The tube was inverted to mix the contents and then allowed to stand until two layers were clearly visible. The clear upper layer containing leukocytes was removed and put in another sterile tube. The leukocytes were washed in sterile PBS twice, by spinning at 600g for 10 min. The leukocyte pellet was resuspended in lysis buffer and RNA extracted in the same way as for fibroblasts (see above).

2.5c. Preparation of plasmid DNA.

Plasmid solution 1: 25% (w/v) sucrose, 50mM Tris pH 8.0, 10mM EDTA pH 8.0.
Plasmid solution 2: 0.2M NaOH, 1% SDS, freshly prepared.
Plasmid solution 3: 3M sodium acetate, pH 4.8, freshly prepared.
L-broth: 10g/L tryptone, 5g/L yeast extract, 10g/L NaCl, pH 7.5; autoclaved.

A single colony of HB101 cells transformed with pUC18(AF3) or pUC18(AF2D) was used to inoculate 5ml of L-broth containing 25μg/ml ampicillin. The culture was incubated at 37^{o}C for 6 hours, after which it was transferred to a large conical flask containing 500ml of L-broth with ampicillin. The 500ml culture was incubated overnight at 37^{o}C with shaking.

The culture was spun in a MSE Coolspin centrifuge at 4,000rpm for 15 min at 4^{o}C. The pellet was resuspended in 20ml of plasmid solution 1. A freshly prepared solution of lysozyme was added to give a final concentration of 1mg/ml. The solution was left at room temperature for 10 min and then 60ml of plasmid solution 2 was added. The solutions were mixed and left on ice for 15 min. 45ml of plasmid solution 3 was then added and mixed. The

solution was left on ice for 60min, before being centrifuged in a Beckman J-2 centrifuge at 4,000rpm for 15min at $4^{o}C$ using a 6 x 250ml rotor. 0.6 volumes of isopropanol was added to the supernatant and the solution was left at $-20^{o}C$ for 60min. It was then spun in a Beckman J-2 centrifuge with a 6 x 250ml rotor at 4000rpm for 30 min at $4^{o}C$ and the pellet was resuspended in 10ml of TE. 1g/ml of caesium chloride and 600µg/ml ethidium bromide was added. The caesium chloride solution was spun at 8,000rpm in a Beckman J-2 centrifuge with a JA20 rotor for 20 min. The resultant supernatant was transferred to a ultracentrifuge tube which was topped up with liquid paraffin. The tubes were heat sealed and centrifuged at 48,000rpm in a Ti 70 rotor in a Beckman L870 ultra centrifuge at $16^{o}C$ for 20hr. The band of plasmid DNA was identified by ultraviolet illumination and extracted from the centrifuge tube with a needle and syringe. The ethidium bromide was removed from the plasmid preparation by repeated extraction with butanol. The solution of plasmid DNA was made 250mM with respect to sodium acetate, pH 4.8, and 0.6 volumes of isopropanol were added. The solution was put in a dry ice-methanol bath for 60min and then left at $-20^{o}C$ overnight. The solution was centrifuged in a Beckman J2 centrifuge in a JA-20 rotor at 10,000rpm at $4^{o}C$ for 30min. The resultant pellet was washed in 70% ethanol and then resuspended in TE and stored at $-20^{o}C$.

2.5d. Southern blot analysis.

i). Restriction enzyme digestion.

10-15µg of genomic DNA was digested with 15 units of the relevant restriction enzyme in the buffer recommended for the particular enzyme by the manufacturer (BRL). Digestions were carried out at $37^{o}C$ for 6-16 hours, for each restriction enzyme except for Taq 1 and Bcl1 which were incubated for one hour at 65^{o} C, and 6 hrs at $50^{o}C$ respectively.

ii). Agarose gel electrophoresis.

10x TBE: 0.09M Tris-borate, 0.002M EDTA.

6x loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water.

DNA which had been digested with a restriction enzyme was electrophoresed through 0.8%-1.0% (w/v) agarose gels containing ethidium bromide (0.5μg/ml) at 1.5 volts/cm. TBE was used as buffer for making the agarose gel and as the running buffer. Lambda DNA fragments produced by restriction enzyme digestion were used as molecular size markers. Each sample was loaded with loading buffer.

Following electrophoresis, the agarose gel was photographed under ultraviolet light with a ruler placed alongside, to determine the distance of migration of the size markers. A standard curve of distance migrated against size of DNA fragment in kilobases was constructed.

iii). Transfer to nylon filters.

Denaturing solution: 1.5M NaCl, 0.5M NaOH

Neutralising solution: 1.5M NaCl, 0.5M tris-HCl pH 7.2 and 0.001M Na_2 EDTA.

20xSSC: 3.0M NaCl, 0.3M $C_6H_5O_7Na_3$.

The method used was essentially that described by Southern, (1975). Briefly, the DNA in the agarose gel was denatured by submerging the gel in denaturing solution and gently shaking for 30 min. The denaturing solution was then replaced with neutralizing solution and left for 60 min with gentle shaking.

The DNA in the agarose gel was then transferred to a nylon membrane (Hybond N, Amersham) by capillary action as follows. A sponge placed in a trough of 20x SSC was overlaid with Whatman 3MM paper soaked in 20x SSC. The gel was placed over this, and the nylon membrane, cut to the same size as the gel, placed on top of the gel. This was finally overlain with two pieces of 3MM paper wetted with 2x SSC and paper towels to an approximate height of six centimeters. Air bubbles between each layer were expelled with a plastic pipette, and plastic film was put around the gel to prevent the transfer solution from bypassing the gel. The assembly was left for approximately 20 hours at room temperature.

Following blotting the positions of the wells in the agarose gel were marked on the membrane. The membrane was rinsed in 2x SSC and was then placed in an oven at $80^{o}C$ for 2 hours to fix the DNA to the membrane.

iv). Preparation of probes.

The fucosidase cDNA probes were donated by Dr. J.S. O'Brien; (for a map of the probes used see fig. 2.1). The fucosidase cDNA probes were cut out of the plasmid vector (pUC18) using the restriction enzyme EcoR1. The fucosidase cDNA was then separated from the plasmid DNA by agarose gel electrophoresis. The band of cDNA was cut out of the gel and the DNA was extracted from the agarose using 'Geneclean' according to the manufacturers instructions. When required probe AB was cut into smaller fragments with other restriction enzymes such as Hinc 11. The fragments were separated on an agarose minigel and the DNA extracted from the agarose using 'Geneclean'.

v). Labelling of the cDNA probes.

Oligolabelling buffer:

Solution O: 1.25M Tris pH 8, 0.125M $MgCl_2$.

Solution A: 5µl of 100mM dATP, 5µl of 100mM dGTP,
5µl of 100mM dTTP, 18µl beta-mercaptoethanol,
100µl solution O.

Solution B: 2M HEPES (titrated to pH 6.6 with 4M NaOH).

Solution C: 90 OD units/ml hexadeoxynucleotide in TE.

Solutions mixed: A : B : C, 20 : 50 : 30.

TE 10mM Tris, 1mM EDTA, pH 7.5 .

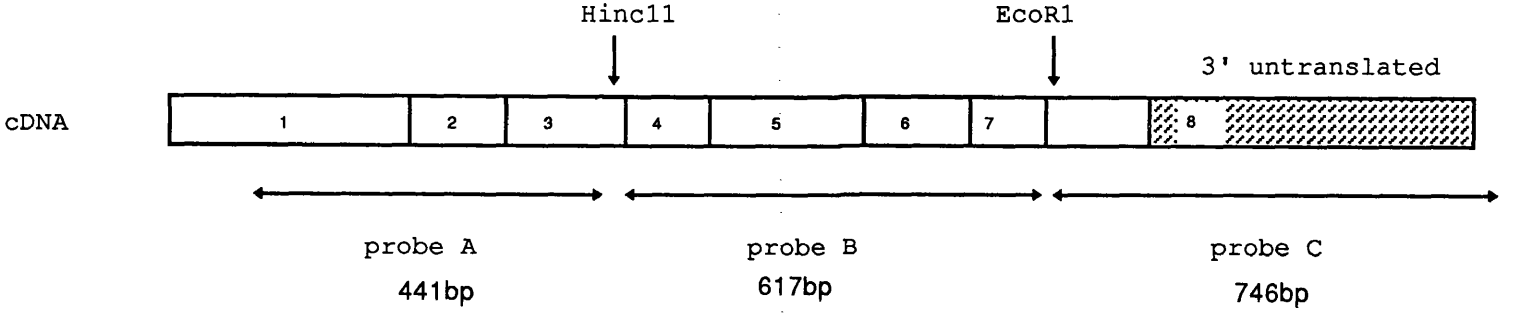


Fig 2.1. Fucosidase cDNA probes.

Random hexanucleotide labelling was used to label the fucosidase cDNA probes with ^{32}P (Feinberg and Vogelstein; 1983). Approximately 30ng of cDNA was denatured by boiling for 5 min and was then put on ice. 1µl of a 10mg/ml solution of bovine serum albumin (BSA) and 5µl oligolabelling buffer were added, followed by 2.5µl of 10µCi/µl of ^{32}P dCTP, and 2 units of Klenow (large fragment of DNA polymerase 1) in a final volume of 25µl. The oligolabelling mixture was left from 6-18 hours at room temperature. The reaction was stopped by addition of 75 µl of TE.

The radiolabelled probe was separated from unincorporated radionucleotides on Sephadex G 50 packed in a Pasteur pipette plugged with sterile glass wool. The column was equilibrated and eluted with TE and 100µl fractions were collected. The fractions containing the peaks of incorporated radionucleotide were identified by use of a Geiger counter. These were pooled and the specific activity measured.

vi). Prehybridization.

100x Denharts solution:

2 g/l (w/v) Ficoll, 2 g/l (w/v) polyvinylpyrrolidone, 2 g/l (w/v) BSA.

Hybridization solution:

4x SSC, 1% (w/v) sodium dodecyl sulphate (SDS), 10x Denharts solution, 50 µg/ml denatured salmon sperm DNA (denatured by boiling).

The nylon membrane to which the DNA had been fixed was wetted in 2x SSC, and then sealed in a polythene hybridization bag. One corner of the bag was cut off to permit addition of the hybridization solution. 10 to 15 ml of solution was added per nylon membrane. All air bubbles were expelled from the bag and the corner was re-sealed. The hybridisation bag was placed in a water bath at $65^{o}C$ for 3 hours.

vii). Hybridization.

The nylon membrane was removed from the bag containing prehybridization solution and sealed in another polythene bag. The radiolabelled probe was denatured by boiling for 5 min, cooled on ice, and then added to the appropriate amount of hybridization solution (10 - 15 ml per nylon membrane). 1×10^6 cpm per ml of hybridization solution was used. The hybridization solution containing the probe was pipetted into the new hybridization bag which was then sealed completely after removal of all the air bubbles. The nylon membrane was hybridized to the probe overnight in a water bath at 65^{o} C.

viii). Washing.

The nylon membranes were placed in a solution of 3 x SSC containing 0.1 % (w/v) SDS on a shaker for 20 min at room temperature. This low stringency wash was repeated twice. This was followed by a higher stringency wash at 65^{o}C for 30 min. The conditions used depended on the probe and the type of DNA to which the probe was hybridiZed. For example, a high stringency wash of 0.8 x SSC containing 0.1 % (w/v) SDS at 65^{o}C was used for cDNA probe A+B with blots of canine DNA, and 0.5 x SSC containing 0.1% (w/v) SDS at 65^{o}C for blots of human DNA.

Following washing, the nylon membrane was wrapped in cling film and placed in an X-ray cassette with one or two intensifying screens, and Kodak X-ray film. The cassette was left at -70^{o}C for 24 hr to a week. The film was developed in an automatic film processor.

2.5. Northern blotting.

i). Denaturing agarose gel preparation.

10xMOPS/EDTA solution:

0.2M MOPS [3-(N-morpholino) propanesulphonic acid], 50mM sodium acetate, 10mM EDTA, pH 7.0, autoclaved.

2g of agarose was dissolved in 20ml of 10 x MOPS/EDTA solution and 174ml of diethylpyrocarbonate (DEPC)-treated autoclaved water in a DEPC- treated and autoclaved flask. The agarose was dissolved by heating in a microwave, and then cooled to 50^{o}C. 10.2ml of 37 % (v/v) formaldehyde was added to the gel solution. The molten gel was then poured into a gel tray with a 20 well comb and allowed to set.

ii). Sample preparation.

Electrophoresis sample buffer:

0.75ml deionised formamide, 0.15ml 10 x MOPS/EDTA, 0.24ml formaldehyde, 0.1ml DEPC-treated water, 0.1ml glycerol, 0.08ml 10 % (w/v) bromophenol blue; freshly prepared.

20μg of total RNA was dissolved in 5μl of DEPC-treated water and 25μl of electrophoresis sample buffer was added . The RNA in the electrophoresis sample buffer was heated at 65^{o}C for 15min. 1μl of ethidium bromide solution (1mg/ml in DEPC-treated water) was then added to each sample, and the samples loaded onto the agarose gel.

iii). Electrophoresis.

The denaturing gels were run at 1.5V per cm for approximately 18 hrs at room temperature. 1 x MOPS/EDTA was used as electrophoresis buffer. Following electrophoresis, the gel was photographed under ultra violet light with a ruler placed alongside the gel.

iv). Transfer of RNA to the filter.

Following electrophoresis, the gel was soaked in 10 x SSC, for two periods of twenty min with gentle shaking. A sponge overlaid with Whatman 3MM paper was placed in a trough of 10x SSC. The gel was placed on the 3MM paper, followed by a piece of nylon membrane, cut to the same size as the gel. This was overlaid by 3MM paper soaked in 10 x SSC and paper towels. Air bubbles were expelled from between each layer using a plastic

pipette. Transfer by capillary action of the RNA in the gel to the nylon membrane was allowed to proceed for approximately 18 hr.

The RNA bound to the membrane was visualised by ultraviolet light transillumination to estimate the efficiency of transfer. The RNA was fixed to the nylon membrane by baking for 2 hours in an oven at 80^{o}C.

v). Prehybridization.

20xSSPE:

3.6M NaCl, 0.2M sodium phosphate, pH 7.7, 0.002M Na_2EDTA.

Hybridization solution:

5x SSPE, 50 % formamide (freshly deionised with Amberlite mixed bed resin), 5x Denharts solution, 0.5% (w/v) SDS, 50υg/ml of denatured salmon sperm DNA.

The nylon membrane was wetted in 2x SSPE and then sealed in a polythene hybridization bag. 12ml of hybridization solution was added to the bag per membrane. The nylon membrane was prehybridized at 42^{o}C for 3 hours.

vi). Hybridization.

The filters were hybridized to fucosidase cDNA probes, which had been radiolabelled by the random hexanucleotide method as for detection of DNA by Southern blotting (2.5d.v). Following prehybridization the nylon membrane was put into another hybridization bag. The radiolabelled probe was denatured by boiling for 5 min, cooled on ice and then added to 12ml of hybridization solution. The hybridization solution containing the probe was added to the hybridization bag, and all bubbles expelled. The bag was sealed completely and placed in a water bath at 42^{o}C for approximately 18 hr.

The nylon membrane was washed in 1x SSC containing 0.1 % (w/v) SDS at room temperature for two periods of 20 min. This was followed by a wash at a higher stringency of 0.1x SSC containing 0.1 % (w/v) SDS at 52^{o}C, for two periods of 20 min.

vii). Autoradiography.

The hybridized nylon membrane was placed in a X-ray cassette with two intensifying screens and Kodak X-Omat film. The cassette was put at -70^{o}C for one to two weeks. The film was developed in an automatic film processor.

2.5f. Amplification by the polymerase chain reaction (PCR).

i). The design of oligonucleotide primers.

Oligonucleotide primers were made by Mr P. Rutland using a DNA synthesis machine (Applied Biosystems), except for primers 4 and 5 which were provided by Dr P. Willems. Primers were designed where possible to have GC:AT ratios of approximately 50:50. Palindromic sequences and complementarity between primer pairs were avoided where possible.

Primer 1: 5' TCC TAC CAT AGT CAG CCT GT (intron 7)
Primer 2: 5' AAA CAG TGA GCA GCG CCT CT (3' non coding)
Primer 3: 5' GGT AGA AAT GAT TGT GAT GTA C (3' non coding)
Primer 4: 5' AAG CTT CCC ATA ACT ACC TCA ACT ACA AAG (exon 7)
Primer 5: 5' TAC TCC AAG CAA ACT CTG CGG GGA CAG CAG (exon 8)
Primer 6: 5' GCT TCA TGC GCG ACA ACT AC (exon 1)
Primer 7: 5' GAT GAG GTG GTA GTA AAT GA (exon 5)
Primer 8: 5' GAA ATG ATT TCA GAT TCT TC (exon 5)
Primer 9: 5' TAA GCA TGA TGC CAG GCT TG (intron 5)
Primer10: 5' AGG AGA TAC CAG TTC CGG AT (intron 6)
Primer11: 5' TCA CAA GTG GGA GAT GTG CA (exon 5)
Primer12: 5' TAT AAA ATA ATA CAT ACT GCA TGT TA (intron 5)
Primer13: 5' CCT GGG GCT ATC GTC GTG AC (exon 5)

Primer 13 was biotinylated at the 5' end. A map of primer sites is shown in fig.2.2.

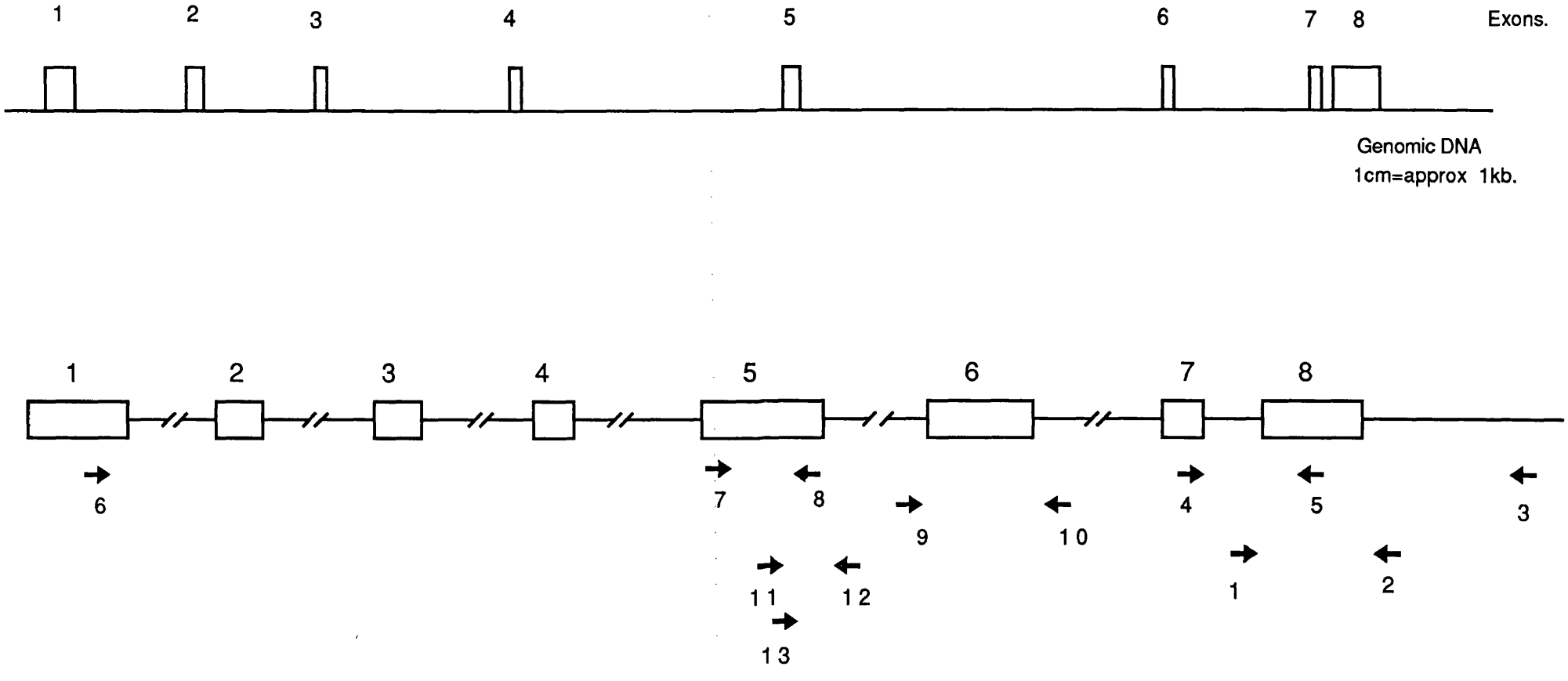


Fig 2.2. Diagram to show the position of primer sequences within the fucosidase gene.

ii). The reaction mixture.

10x PCR buffer: 500mM KCl, 100mM Tris-Cl pH 9.0, 15mM $MgCl_2$, 0.1% gelatin, 1% Triton X-100.

The reaction mixtures contained: 200μM dATP, 200μM dCTP, 200μM dGTP, 200μM dTTP, 1x PCR buffer, 50pmol of each oligonucleotide primer and 500ng of DNA in a total volume of 100μl. The reaction mix was made up to 100μl with sterile water and overlaid with 70μl of mineral oil. A negative control consisting of all the reaction components except the DNA was set up each time.

iii). The PCR reaction.

The reactions were carried out on a Techne PHC-2 machine and according to the method of Saiki et al., (1985). All the components of the reaction, with the exception of the Taq polymerase, were added to sterile 500μl Eppendorf tubes. The tubes were placed in the Techne machine and heated to 95°C for 10 min. The tubes were then cooled (in the machine) to the appropriate annealing temperature for the primers being used and 2.5 Units of Taq polymerase (Promega) was added to the tubes whilst in the machine. The machine was programmed to perform a number of cycles (25-30) of heating at three different temperatures for defined periods for primer extension, denaturation and annealing. This was followed by a final extension step of ten minutes at 72°C. The annealing temperature and incubation times chosen were specific for each pair of primers:

Primer 1+2: 72°C, 2 min; 95°C, 1 min; 60°C, 1.5 min; 30 cycles.

Primer 1+3: 72°C, 1.5 min; 95°C, 50 sec; 55°C, 1 min; 30 cycles.

Primer 4+3: 72°C, 1.5 min; 95°C, 50sec; 55°C, 1min; 30 cycles.

Primer 4+5: 72°C, 1.5 min; 95°C, 50 sec; 45°C, 1min; 30 cycles.

Primer 6+2: 72°C, 2.5 min; 95°C, 1 min; 60°C, 2 min; 35 cycles.

Primer 7+8: 72°C, 3 min; 95°C, 1 min; 58°C, 2 min; 30 cycles.

Primer 9+10: 72°C, 2 min; 95°C, 1 min; 55°C, 1.5 min; 30 cycles.

Primer 11+12: 72°C, 1.5min; 95°C, 1 min; 55°C, 1.25min; 30 cycles.

Primer 12+13: 72°C, 1.5min; 95°C, 1 min; 53°C, 1.25min; 30 cycles.

iv). Restriction enzyme digestion of amplified products.

20% of the amplified product was digested with the relevant restriction enzyme (15 Units) in the buffer supplied by the manufacturer (BRL). The reaction was incubated for 3 hours at 37°C, except for Taq 1 which was incubated at 65°C for 1 hour.

v). Analysis of PCR products.

Agarose gel electrophoresis

15% of the amplified product was analysed by electrophoresis in a 3% mini gel (1 % agarose and 2 % Nusieve agarose) stained with ethidium bromide (0.5μg/ml). Lambda phage size markers were run on the same gel which was photographed under ultraviolet light.

Non denaturing polyacrylamide gel electrophoresis.

6x loading buffer: 50% glycerol, 0.25% xylene cyanol, 0.25% bromophenol blue, in water.
40% acrylamide (19:1): 38g acrylamide, 2g N,N'-methylene bis acrylamide in 100ml water.

40% of the PCR product was analysed by electrophoresis in polyacrylamide gel (19:1), using a 10% resolving gel and 5% stacking gel in TBE. The samples were applied in loading buffer, and electrophoresis was carried out at 5 volts/cm. Following electrophoresis, the gel was stained with ethidium bromide (0.5μg/ml) and photographed under ultraviolet light.

2.5g).Chemical mismatch analysis.

Chemical mismatch analysis was carried out essentially according to the method of Montanadon et al., 1989.

i). Probe preparation.

Labelling of the probe at the 5' end.

10x polynucleotide kinase buffer: 0.5M Tris-Cl pH 7.6, 0.1M $MgCl_2$ 50mM dithiothreitol, 1mM spermidine, 1mM EDTA pH 8.0.

DNA to be used as the probe was amplified by PCR and 20% of the product was analysed by electrophoresis on an agarose mini gel stained with ethidium bromide. The band of amplified product was excised from the gel under ultraviolet light. The DNA was extracted from the gel slice using 'Geneclean'. The probe DNA was end labelled with ^{32}P using T4 polynucleotide kinase in the following reaction: 1x polynucleotide kinase buffer, 50ng of amplified product DNA, 15μCi of ^{32}P dATP and 10 units of T4 polynucleotide kinase. The reaction was incubated at 37°C for 30 min.

The labelled amplified product was separated from unincorporated nucleotides by gel filtration on Sephadex G-50 equilibrated with TE pH 7.8. 50μl fractions in TE were collected and those containing the peak of incorporated radionucleotide (determined by use of a Geiger counter) were pooled.

The incorporation of the radionucleotide into the probe was estimated by spotting 1μl of the pooled fractions onto four DE-81 filters. Two of the filters were washed with 0.5M sodium phosphate (3 x 5 min), water (1 x 5 min), and ethanol (1 x 1 min). The specific activity of the two washed, and two unwashed filters was then counted in a scintillation counter.

Labelling of the probe by PCR.

The probe DNA was also labelled by incorporation of ^{32}P α dCTP during the PCR, using the following reaction mixture: 1x PCR reaction buffer, 500ng DNA, 50 pmol of each primer, dGTP, dTTP and dATP at a concentration of 200μM and dCTP at a concentration of 190μM and 10μCi of ^{32}P α dCTP. The amplification cycle used was as above (2.5f).

The amplification product was initially separated on a Sephadex G-50 column. 50μl fraction in TE were collected. Fractions containing incorporated radionucleotide were pooled and the specific activity counted.

Alternatively, the amplified product was purified by electrophoresis through an agarose minigel stained with ethidium bromide. The band of amplified product was cut out of the gel, under uv illumination, and the DNA extracted from the agarose by 'Geneclean', according to the manufacturers instructions.

ii). Preparation of unlabelled 'mutant' DNA.

Genomic DNA was amplified using the same primers and PCR cycle conditions as for the probe. The products were purified by electrophoresis in agarose as described previously.

iii). Heteroduplex formation.

Sterile siliconised Eppendorf tubes were used for heteroduplex formation and all subsequent steps. 5ng of labelled probe was mixed with 100ng of unlabelled mutant PCR product in 0.1M Tris-HCl pH8.0 buffer containing 0.3M NaCl in a final volume of 10μl. The annealing mixture was overlaid with 10μl of mineral oil, and denatured in a boiling water bath for 5 min. The tubes were then placed in a water bath at 65^{o}C for 18 hours. The annealing mixes were placed on ice and 30μg of mussel glycogen was added to each tube (as a carrier for DNA precipitation). The DNA was precipitated by adding 200μl of 0.3M sodium acetate/0.1M EDTA followed by 750μl of ethanol. The tubes were placed in a methanol-dry

ice bath for 15 min, and then spun in a microfuge for 10 min. The pellets were washed twice with 70 % (v/v) ethanol and then resuspended in 7μl TE (for hydroxylamine modification) or 9μl TE (for osmium tetroxide modification).

iv).Modification with hydroxylamine.

20μl of a freshly prepared 4M solution of hydroxylamine adjusted to pH 6 with diethylamine, was added to 7μl of heteroduplex. The heteroduplexes were incubated at 37°C for 2 hours. They were then precipitated by addition of 200μl of 0.3M sodium acetate /0.1M EDTA and 750μl of ethanol, put in a dry ice-methanol bath for 15 min, and then spun in a microfuge for 10 min. The pellets were washed twice in 70 % ethanol.

v). Modification with osmium tetroxide.

Osmium tetroxide solution: 5mM Tris-Hcl pH 8, 0.5mM EDTA, 3 % pyridine, 0.025 % osmium tetroxide.

25μl of freshly made osmium tetroxide solution was added to 9μl of heteroduplex. The heteroduplexes were incubated for 2 hours at 37°C. They were then precipitated with ethanol as for hydroxylamine modification.

vi). Cleavage with piperidine.

The pellets of heteroduplex DNA were resuspended in 50μl of a IM piperidine solution by vortexing for 45 seconds. They were then incubated in a heating block at 90°C for 30 min. The DNA was then precipitated with ethanol as for hydroxylamine modification.

vii). Denaturing polyacrylamide gel electrophoresis.

40% acrylamide (19:1): 38g acrylamide, 2g N,N'-methylene bis acrylamide in 100ml water. Denaturing gel composition (8%): 75.6g urea, 27ml 40% acrylamide, 18ml 10x TBE, made up to 180 ml; filtered. 1ml 10% ammonium persuphate and 112μl TEMED were added just before the gel was poured.

Formamide loading buffer: 95 % formamide, 20mM EDTA, 0.05 % bromophenol blue, 0.05 % xylene cyanol.

The pellets were resuspended in 5µl TE and 5µl formamide loading buffer was added. The samples were denatured by boiling for 3 min and then analysed on a 8% denaturing polyacrylamide gel. The polyacrylamide gel was fixed in 12% (v/v) methanol, 10% (v/v) acetic acid in water for 30min, and then dried in a vacuum drier. The dried gel was autoradiographed overnight at -70^{o}C.

2.5h. Direct sequencing of PCR products.

i). Template preparation.

Genomic DNA was amplified by PCR using 50 pmol of each primer as above (2.5f), to generate a double stranded product. 15% of the amplified product was analysed by electrophoresis in an agarose mini gel. The remaining 85 % of product was extracted with phenol/chloroform isoamyl alcohol (24:1), and then chloroform isoamyl alcohol (24:1). The DNA was precipitated by addition of an equal volume of 4M ammonium acetate and four times its volume of isopropanol. It was left at room temperature for 15 min and finally spun at 12,000g for 10 min. The pellets were washed twice with 70 % ethanol.

The double stranded PCR product was amplified in a PCR using 50pmol of only one of the primers, to generate some single stranded PCR product. 15% of the product was checked by electrophoresis on an agarose minigel. The remaining product was extracted with phenol/chloroform and chloroform and then precipitated with ammonium acetate/isopropanol (as above). Following two washes with 70 % ethanol the pellets were resuspended in 10µl of sterile water.

The two strands of PCR product generated by amplification with primers 12 and 13 were separated using streptavidin-coated magnetic beads (Dynabeads) according to the manufacturers instructions, prior to sequencing.

ii). Sequencing reaction.

The single stranded PCR products were sequenced using the dideoxy chain termination method (Sanger et al., 1977) with a Sequenase kit (USB).

ii) a. Annealing template and primer.

Reaction buffer (5x concentrated): 200mM Tris-HCl pH 7.5, 100mM $MgCl_2$, 250mM NaCl

The primer used in the initial PCR to generate a double stranded product, but not in the subsequent reaction to generate single stranded products, was used as the sequencing primer. The annealing reaction contained: 0.5pmol of primer, PCR product and 1x reaction buffer in a total volume of 10µl. The Eppendorf tube containing the reaction mixture was heated to 65^{o} C for two min, then placed in a small beaker of water at 65^{o}C, which was allowed to cool from 65^{o}C to 30^{o}C over 30 min. The tube was then put on ice.

ii)b. Labelling reaction.

Labelling mix (5x concentrated): 7.5µM dGTP, 7.5µM dCTP, 7.5µM dTTP.

The following labelling reaction mixture was made: 10µl of template-primer, 1µl of 1.0M dithiothreitol (DTT), 2µl of labelling mix and 0.5µl of [-^{35}S]dATP, on ice. 2µl of 1:8 diluted Sequenase version 2 enzyme (USB) was then added, and the mixture left at room temperature for 4 min.

ii)c. Termination reaction.

ddGTP termination mix: 80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddGTP, 50mM NaCl;

ddATP termination mix: 80µM dGTP, 80µM dATP, 80µM dTTP, 80µM dCTP, 8µM ddATP, 50mM NaCl;

ddTTP termination mix: 80µM dGTP, 80µM dATP, 80µM dTTP, 80µM dCTP, 8µM

ddTTP, 50 mM NaCl;

ddCTP termination mix: 80μM dGTP, 80μM dATP, 80μM dCTP, 80μM dTTP, 8μM ddCTP, 50mM NaCl.

Stop solution: 95 % formamide, 20mM EDTA, 0.05 % bromophenol blue, 0.05 % xylene cyanol.

3.5μl of labelling reaction was added to four prewarmed tubes containing ddGTP, ddATP, ddCTP, or ddGTP termination mixes. The tubes were incubated at 37°C for 5 min. The reaction was stopped by addition of 4μl of stop solution.

iii). Polyacrylamide gel electrophoresis

3μl of each of the sequencing reaction mixtures was denatured by boiling for 2 min and then loaded on a 6% denaturing polyacrylamide gel (see 2.5v ii).). The sequencing reactions were electrophoresed for approximately 2 hours at 60 watts. The gel was then fixed in a solution of 12 % methanol, 10 % acetic acid in water, for 30 min. Following this, the gel was dried under vacuum on a piece of 3MM filter paper. The dried gel was put in an X-ray cassette with some X-ray film and autoradiographed at -70°C for approximately 18 hours.

2.5i. Reverse transcription of RNA.

The method used was basically that of Kawasaki, (1989). Total RNA extracted from fibroblasts or leukocytes was reverse-transcribed into cDNA in the following reaction: 3μg of total RNA was put in a DEPC-treated and autoclaved Eppendorf tube together with 100 pmoles of random hexanucleotides. The tube was heated at 65°C for 2 min and then put on ice. The following were then added: 20units of RNasin, 1.5mM $MgCl_2$, 10mM DTT, 1mM of each dNTP, PCR buffer (Promega), and 56 units of reverse transcriptase (Promega). The reaction was incubated at 37°C for 60 min. The reaction was stopped by addition of 80μl of TE, followed by heating to 65°C for 5 min.

One tenth of this product was used in a PCR with primers 6 and 2, specific for exon 1 and for the 3' non coding region, respectively (see fig. 2.2). The reaction mixture consisted

of: 10µl of cDNA, 10µl of 10x PCR buffer, 2µl of 10mM dNTPs, 50 pmol of each primer, in a total volume of 100µl.The reactions were analysed by running 15 % of the product on an agarose minigel stained with 0.5µg/ml ethidium bromide.

2.6. Clinical details of the patients.

Patient S.B.

S.B. was born following a full term normal delivery in Zambia to distantly related Asian parents. Her birth weight was 3.8kg. There were 5 normal older siblings. Her early milestones were normal. She smiled at 6 weeks, sat unsupported at 8 months and at 16 months was walking steadily and used three words appropriately. From the age of 18 months she gradually became uninterested in toys, commenced mouthing and casting objects and her language skills deteriorated to grunts and pointing. Her gait became progressively more unsteady.

The family moved to the U.K. in 1989. At 6 1/2 years S.B. had no words but waved 'bye-bye' and pointed to indicate her needs. She could finger-feed a biscuit but could not use a spoon. She walked on a broad based gait and fell backwards after every few steps. She was not toilet trained. Her weight and height were below the 3rd centile. Head circumference was on the 5th centile. She had coarse facial features, protruding tongue, kyphosis, contracture of the right elbow and hirsuitism. There was no hepatosplenomegaly or skin lesions. Breathing was noisy due to upper airway obstruction and a Harrison's sulcus was visible. She had a thin muscle bulk with normal tone and reflexes. She continuosly mouthed and cast objects and had no constructive play. There was no corneal clouding and vision was normal. Distraction testing revealed hearing thresholds elevated at 50dB bilaterally with flat tympanograms.

Currently, aged 8 years, she attends a school for children with severe learning difficulties. Her walking has deteriorated further. She wears a helmet to prevent head injury and arm splints to deter hand chewing.

A first cousin died in Zambia aged 4 years. She is reported to have had identical physical and developmental problems as S.B.

Patient A.M.

A.M. was born to unrelated parents from Bangladesh. His early development was normal. He sat at 6 months and walked without support at 2 yr. He could feed himself with both hands and spoke a few words. He lost the ability to walk at 4 yr and to stand and sit at 4-6 yr. At 7 yr he had a febrile illness with dehydration and two convulsions

after which further deterioration was seen. At the age of 10 yr he was severely mentally retarded and was abnormal looking. He had joint contractures at elbows, wrists and knees and marked kyphosis. Angiokeratoma corporis diffusum was present at 10 yr. He was still living at 15 yr.

His mother had one miscarriage and one daughter with multiple congenital abnormalities at 39 days, prior to the birth of A.M. A sibling of normal phenotype has recently been born, following prenatal testing for fucosidosis.

Patient C.L.

The clinical details of C.L. were reported by Primrose, (1972), and MacPhee and Logan, (1977). The patient was born following a normal pregnancy and delivery in 1951. Her birth weight was 3.6 kg. The father of the patient has never been identified and the paternal grandfather cannot be excluded. His parents were first cousins. The patient's early development was slow but she learnt to sit and walk and speak a few words. She developed a skin rash at 5 yr and was referred to an orthopaedic surgeon at the age of 11 yr because of deterioration in her ability to walk. At the age of 20 yr she had moderate flexion contracture of the legs and severe dorsal scoliosis but no gross kyphosis. She had coarse facies. She had occasional single words of indistinct speech and understood some of what was said to her. An extensive red maculo-papular rash was present with maximal distribution over the pelvic region. She was still alive at 38 yr.

Patient V.O.

V.O. was diagnosed as having fucosidosis at the age of 2 yrs. At present, at 11 yrs of age, she is moderately dysmorphic and only slightly mentally retarded. She walks and talks normally and attends a normal school. She is of English descent. Her parents are unrelated.

Clinical details of patients C.B. and G.J. were not available.

Fucosidosis at the protein level.

3.1. Introduction.

Typically fucosidosis patients have negligible α-L-fucosidase activity in all tissues. Assay of fucosidase activity in leukocytes, fibroblasts or plasma is a convenient rapid method for diagnosis of fucosidosis which is used routinely. Diagnosis based on enzyme activity can be complicated, however, by a low plasma activity polymorphism which is found in approximately 8% of the normal population. Various atypical patients have been described, some of which may be misdiagnosed and have a reduced fucosidase activity as a result of the low activity polymorphism.

The defect was investigated at the protein level in six patients previously described as having fucosidosis.

3.2. α–L-fucosidase activity.

The fucosidase activity of fibroblasts , leukocytes or plasma from fucosidosis patients, heterozygotes and controls was assayed using the synthetic substrate 4-methylumbelliferyl-α-L-fucopyranoside. The results are presented in tables 3.1 to 3.4.

3.2a. Patients with activity below 1% of the control mean.

As can be seen from Table 3.1, four of the fucosidosis patients in the study (S.B., G.J., C.L., and A.M.) had negligible fucosidase activity in fibroblasts or leukocytes (<1% of the control mean). This is typical of fucosidosis patients. Willems et al. (1991) have reported the mean fucosidase activity in fibroblasts (43 patients) or leukocytes (47 patients) to be 1 +3% of control mean.

	Fibroblasts. (nmol/hr/mg)	% of control mean.	Leukocytes. (nmol/hr/mg)	% of control mean.
CONTROLS.				
(n=5)	124.5∓31.6	100%	(n=4) 77.5∓8.6	100%
PATIENTS.				
C.B.	47.1	37.80%	ND	
S.B.	ND		0	0
G.J.	0.59	0.47%	ND	
C.L.	0.42	0.34%	0	0
A.M.	0.46	0.37%	0	0
V.O.				
2yr	44	35.30%	a) 19.6	25.30%
			b). 6.3	8.10%
10yr			69.1	89%
11yr			146	188.40%

Table 3.1. Alpha-L-fucosidase activity of the fucosidosis patients.

	Leukocyte.					Plasma.			
	(nm/hr/mg)	% of control mean.	Fuc/Gal	Fuc/Man	Fuc/Hex	nmol/hr/mg	% of control mean.	Fuc/Hex	Fuc/Man
CONTROLS.									
(n=4)	77.5∓8.6	100%	0.33	0.47	47.5	(n=2)713.4∓397.	100%	0.83	11.9
PATIENT									
S.B.	0	0				24.3	3	0.03	0.5
HETEROZYGOTES									
Mother of S.B.	39	50.3	0.23	0.21	26	1113	156	0.85	11.1
Father of S.B.	30	38.7	0.17	0.14	18.7	781	109.5	0.73	12.1
SIBS OF S.B.:									
D.B.	53	68.4	0.28	0.58	37.8	845.8	118.5	0.86	12.2
N.B.	22	28.4	0.17	0.22	18.3	1082	152	0.92	12.1
Sa.B.	37	47.7	0.23	0.32	28.5	857	120.1	0.78	8.6
H.B.	23	29.7	0.15	0.22	19.2	1022	143.2	1.05	10.2
A.B.	24	31	0.17	0.24	18.5	736	103.2	0.81	8.8

Fuc; alpha-L-fucosidase Man; alpha-D-mannosidase Gal; beta-galactosidase Hex; beta-hexosaminidase

Table 3.2. Alpha-L-fucosidase activity of the family of patient S.B.

	Fibroblasts. (nmol/hr/mg)	% of control mean.		Leukocytes. (nmol/hr/mg)	% of control mean.
CONTROLS.					
(n=4)	124.5∓31.7	100%	(n=4)	77.5∓8.6	100%
PATIENT.					
A.M.	0.46	0.37%		0	0
HETEROZYGOTES.					
Mother of A.M.	44.7	35.90%		ND	
Father of A.M.	72	57.80%		ND	
Sib of A.M.	ND			33	42.60%

Table 3.3. Alpha-L-fucosidase activity of the family of patient A.M.

	Leukocyte. (nmol/hr/mg)	% of control mean.		Plasma. (nmol/hr/mg)	% of control mean.
CONTROLS.					
(n=4)	77.5∓8.6	100%	(n=2)	713.4∓396.7	100%
PATIENT.					
V.O.					
2yr	19.6	25.30%		224	31.40%
	6.3	8.10%			
10yr	69.1	89.20%			
11yr	146	188.40%			
HETEROZYGOTES.					
Mother of V.O.	97	125.20%		212	29.70%
Father of V.O.	164.9	212.70%		504	70.60%

Table 3.4. Alpha-L-fucosidase activity of the family of patient V.O.

The parents of patient S.B. had leukocyte α-L-fucosidase levels intermediate between controls and the patient, as expected for an autosomal recessively inherited disease (Table 3.2). The fucosidase activity of the sibling D.B. was well above that of the parents (who are obligate heterozygotes). D.B is therefore probably homozygous for the normal allele. The siblings N.B., H.B., and A.B all had leukocyte fucosidase activities below that of the parents but above that of the patient S.B. These three siblings are probably therefore heterozygous for the normal fucosidase allele and the mutant allele. Sa.B had leukocyte fucosidase activity above that of the siblings N.B., H.B., and A.B., but just below that of the mother. On the basis of these enzyme activity results, she also would appear to be heterozygous. The ratio of α-L-fucosidase to α-D-mannosidase activity of D.B. was slightly higher than the control mean, whereas that of the parents of S.B., and N.B., H.B., and A.B. were similar and approximately half that of the control mean. The enzyme activity ratio of the sibling Sa.B. was above that of the parents and three siblings. On the basis of fucosidase to mannosidase activity ratio she would be designated homozygous for the normal allele. An increase in mannosidase activity has been observed in fucosidosis patients (Matsuda et al., 1973). As can be seen from table 3.2 carrier detection on the basis of plasma fucosidase activity is not possible.

The parents of A.M. had enzyme activities intermediate between controls and the patient A.M., as expected. The sibling of A.M. also had intermediate fucosidase activity and is therefore also heterozygous for the mutant allele (Table 3.3).

Material was not available from the families of G.J. or C.L.

3.2b. Patients with fucosidase activity above 1% of the control mean.

The patient C.B. had intermediate fucosidase activity in fibroblasts (47.1 nmol/hr/mg, 37.8% of control mean; Table 3.1). This value is higher than activities typically present in fibroblast extracts of fucosidosis patients (Willems et al. 1991). A low plasma fucosidase enzyme activity polymorphism is found in approximately 8% of the normal population with

activity 10 - 30% of the control mean. Variants also have a lower activity in cultured fibroblasts - less than 50% of the control mean (Van Elsen et al. 1983). The low activity polymorphism does not appear to be expressed in leukocytes, however. Deficient activity in leukocytes is consequently a more reliable assay for the diagnosis of fucosidosis. Unfortunately leukocytes were not available from this patient.

At the age of two years the patient V.O. had low leukocyte activity (19.6 and 6.3 nmol/hr/mg, 25.3% and 8.1% of control mean), and intermediate fibroblast activity (44 nmol/hr/mg, 35.3% of control mean; Table 3.1). Plasma activity was also intermediate (224.0 nmol/hr/mg, 31.4% of control mean). These three values are all above activities typically found in fucosidosis patients. When ten years of age her leukocyte activity was within the normal range (69.1 nmol/hr/mg, 89.0% of control mean). At eleven years leukocyte activity had increased further (146 nmol/hr/mg, 188.4% of control mean). At this time her clinical regression was reported to have slowed. The parents of V.O. both had leukocyte fucosidase activities within the normal range (Mother V.O., 97.0 nmol/hr/mg; Father V.O., 164.9 nmol/hr/mg; 125.2% and 212.7% of control mean respectively, Table 3.4). These activities are above levels typical for obligate heterozygotes for fucosidosis (52 + 18% of control mean in leukocytes (n=23); Willems et al. 1991).

The patient V.O., therefore, appears to have had a partial deficiency of fucosidase activity in fibroblasts and leukocytes when aged two. However, by eleven years of age the activity in her leukocytes had increased to normal levels. The patient V.O. is clearly atypical of fucosidosis patients. To investigate further the molecular basis of the defect in this atypical patient, the residual fucosidase activity was characterised.

3.3. Characterization of fucosidase from fibroblasts of patient V.O.

To characterise the residual fucosidase activity in fibroblasts of patient V.O., its pH optimum, Km, thermostability and immunoprecipitation equivalence point were investigated. The fibroblasts were taken from the patient at 2yrs of age.

3.3a. pH-dependence of activity.

The fucosidase activity in fibroblasts from a control and patient V.O. was assayed at different pHs ranging between pH 3 and 7.5 (fig. 3. 1). As can be seen from the graph, the control fucosidase has a broad activity range with a pH optimum at pH 4.5 and a shoulder at approximately pH 6. The pH activity-profile for the activity in the fibroblasts of patient V.O. has the same pH optimum at pH 4.5 as the control enzyme but shows a more marked second pH optimum at pH 6. The control enzyme has a relatively higher activity at acidic pH (at pH 4.0 the control enzyme has 90% of maximum activity, whereas that of V.O. has 73% activity), and a relatively lower activity at higher pH (at pH 6.5 the control enzyme has 70% maximum activity while that of V.O. has 90% activity). Fucosidase can be separated into an aggregated form, type 1 and a monomeric form, type 2, on the basis of physical properties. Type 1 and 2 have 75% and 15% of the maximum activity when assayed at pH4.0, respectively (Robinson and Thorpe 1973), possibly due to the lower stability of the monomeric form at acidic pH. These results suggest that at a low pH a relatively higher proportion of the enzyme from the patient V.O. is in the monomeric form than in the control enzyme. This may indicate a decreased tendency of the subunits to aggregate.

3.3b. Km determination.

The fucosidase activity of fibroblasts from a control and patient V.O. was determined using a range of different substrate concentrations, between 0.1 and 1.0 mM at pH 4.5 (fig. 3.2). The values of Km were calculated using a linear regression program to obtain the line of best fit to a plot of 1/v against 1/[s] (Lineweaver-Burk).

	Km
Control 1	140υM
Control 2	48υM
V.O.	44υM ; 46υM

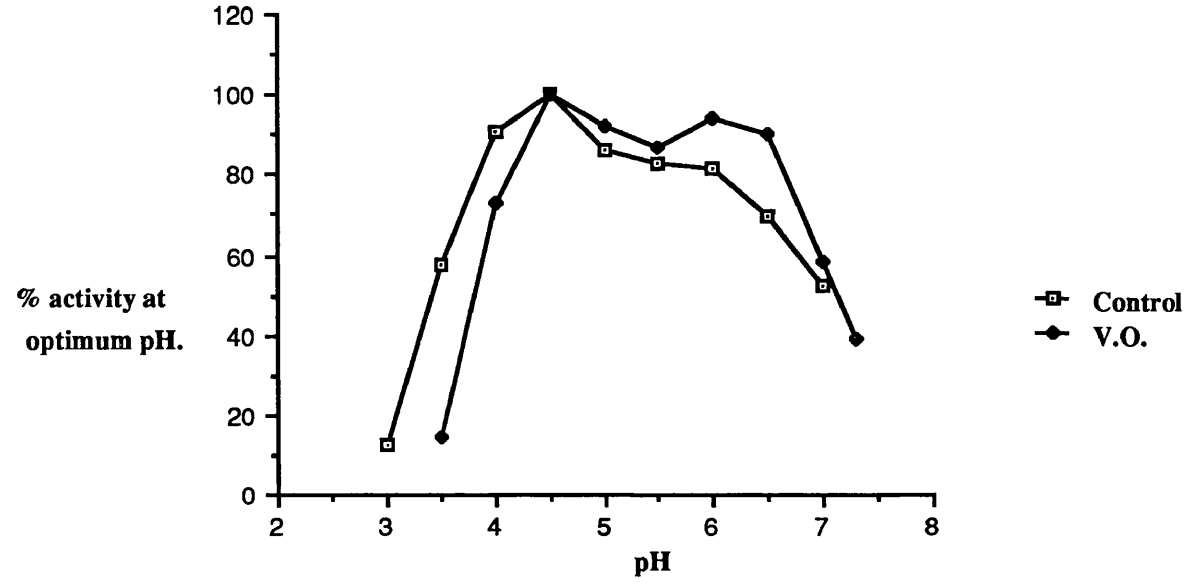


Fig.3.1. pH dependence of activity.

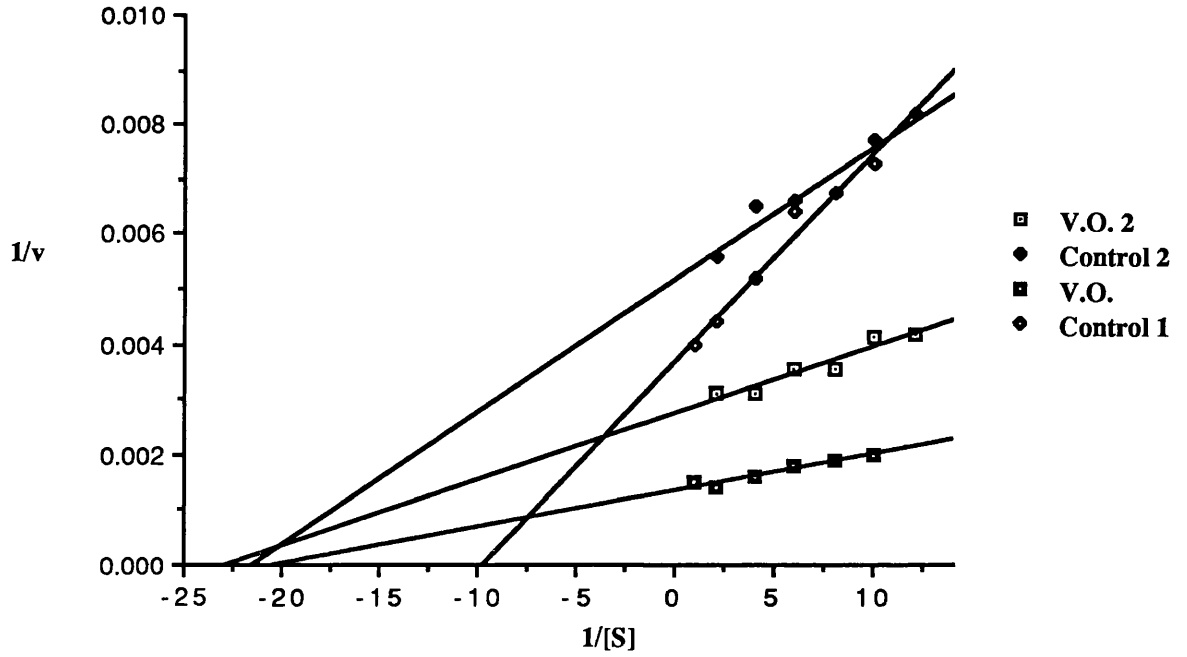


Fig.3.2. Lineweaver-Burk plot for alpha-L-fucosidase.

The Km of V.O. fibroblast fucosidase was not appreciably different from that of the control fibroblast enzyme. Km values for control plasma and liver fucosidase using 4MU as substrate have been reported as being 53υM and 83υM respectively (Willems et al. 1981; Robinson and Thorpe, 1974).

3.3c. Heat inactivation.

Fibroblast extracts from patient V.O. and controls were preincubated at temperatures ranging from $0^{o}C$ to $60^{o}C$ at neutral pH before assay of their fucosidase activity at pH 4.5 (fig 3.3). The enzyme from patient V.O. was more stable at higher temperatures of preincubation than the control; at $50^{o}C$ preincubation 55% activity and 99% activity remained in control and V.O. fibroblast extracts respectively. At temperatures below this heat stability was not appreciably different. At the pH of preincubation most of the enzyme would be in the monomeric form (Thorpe and Robinson, 1978).

3.3d. Immunoprecipitation.

Fibroblast extracts from the patient V.O. and a control were incubated with a range of different dilutions of polyclonal goat anti-(human α-L-fucosidase) serum. The antigen-antibody complex was precipitated and the fucosidase activity remaining in the supernatant was assayed, (Fig 3.4).

The equivalence point for the precipitation of fucosidase activity was 25,000 and 35,000 for the control and patient V.0. respectively. These results show that the fucosidase enzyme from the fibroblasts of patient V.O. is recognised by the polyclonal antibodies used.

3.4. Thin layer chromatography (TLC) of urines.

Urine samples were available from three of the fucosidosis patients: S.B., A.M., and V.O., taken when she was ten years of age. The total sugar concentration of urine per nmol of

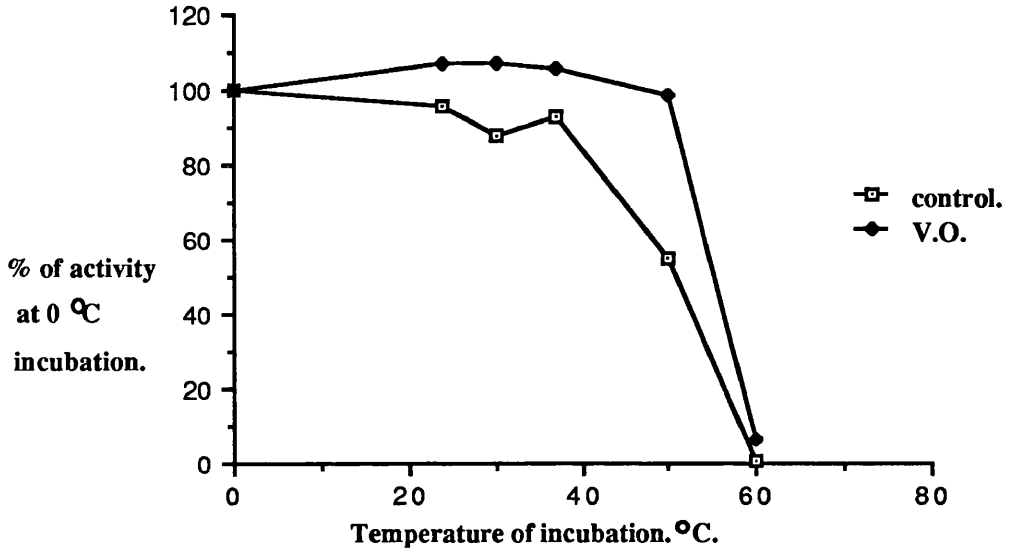


Fig.3.3. Heat inactivation of alpha-L-fucosidase.

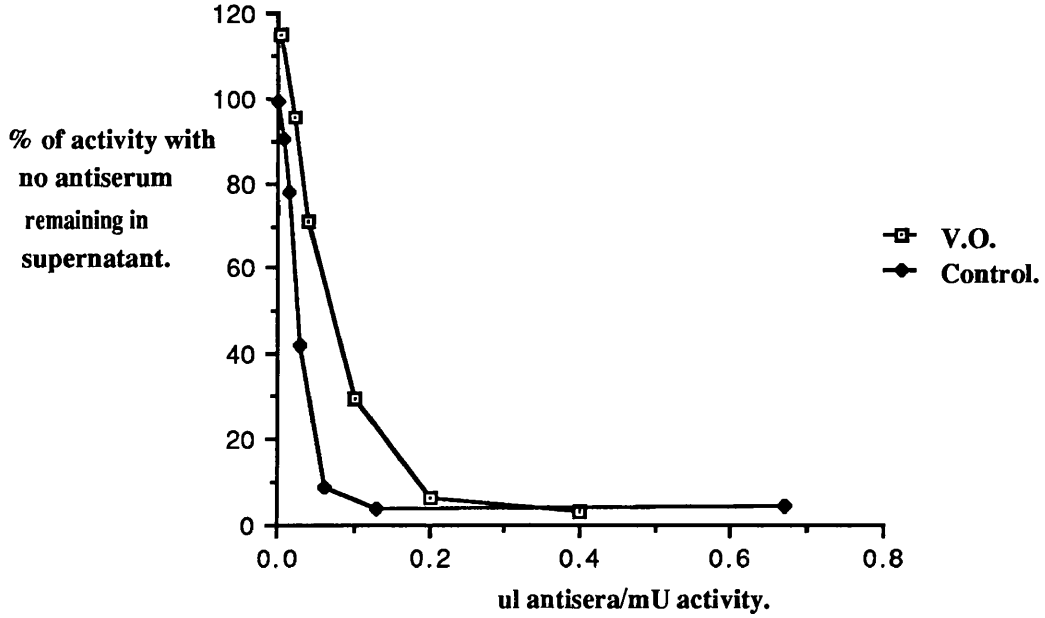


Fig.3.4. Immunoprecipitation of fucosidase.

creatinine was estimated.

	mg fucose/nmol creatinine
Control	0.2
Patient A.M.	0.6
Patient V.O.	0.26

The total sugar was higher in the fucosidosis patient A.M., than in controls, as expected. It was only slightly higher in the atypical patient V.O.

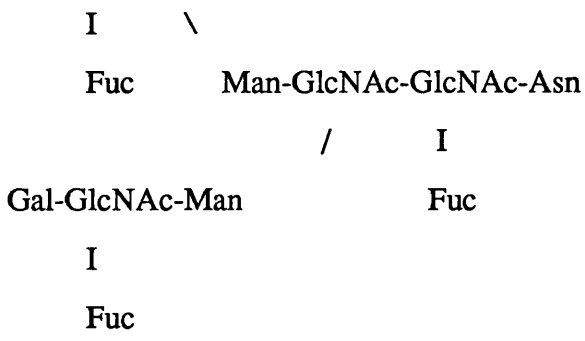
The urines were analysed by TLC in order to investigate the excretion of storage products. Samples were deionised, freeze-dried, and resuspended in deionised water to achieve equal creatinine concentrations. The plates were stained with orcinol to detect oligosaccharides.

As can be seen from fig 3.5a and b, the fucosidosis patients A.M. and S.B. have high molecular weight storage products excreted in the urine which are absent from control urine. The patients S.B. and A.M. have storage products which comigrate with the fucoglycoasparagine markers FG2, FG6, FG7, and FG8 (see fig 7.2 for the structure of the markers).

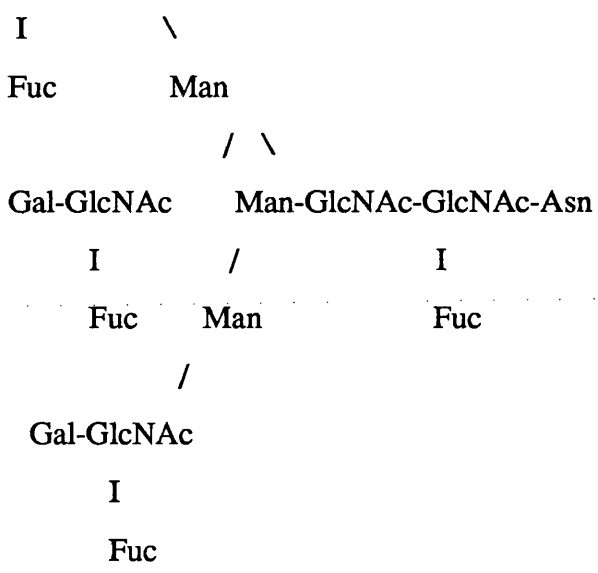
FG2. Gal-GlcNAc-Man-Man-GlcNAc-OH
I
Fuc

FG6. Gal-GlcNAc-Man-Man-GlcNAc-GlcNAc-Asn
I I
Fuc Fuc

FG7. Gal-GlcNAc-Man



FG8. Gal-GlcNAc



A fifth low molecular band which migrated further than Fuc-GlcNAc-Asn (marked with an asterisk), was also present in the urine of A.M. and S.B., but not control urine.

High molecular weight storage products were absent from the urine of patient V.O. No high molecular weight storage products were observed even when double the

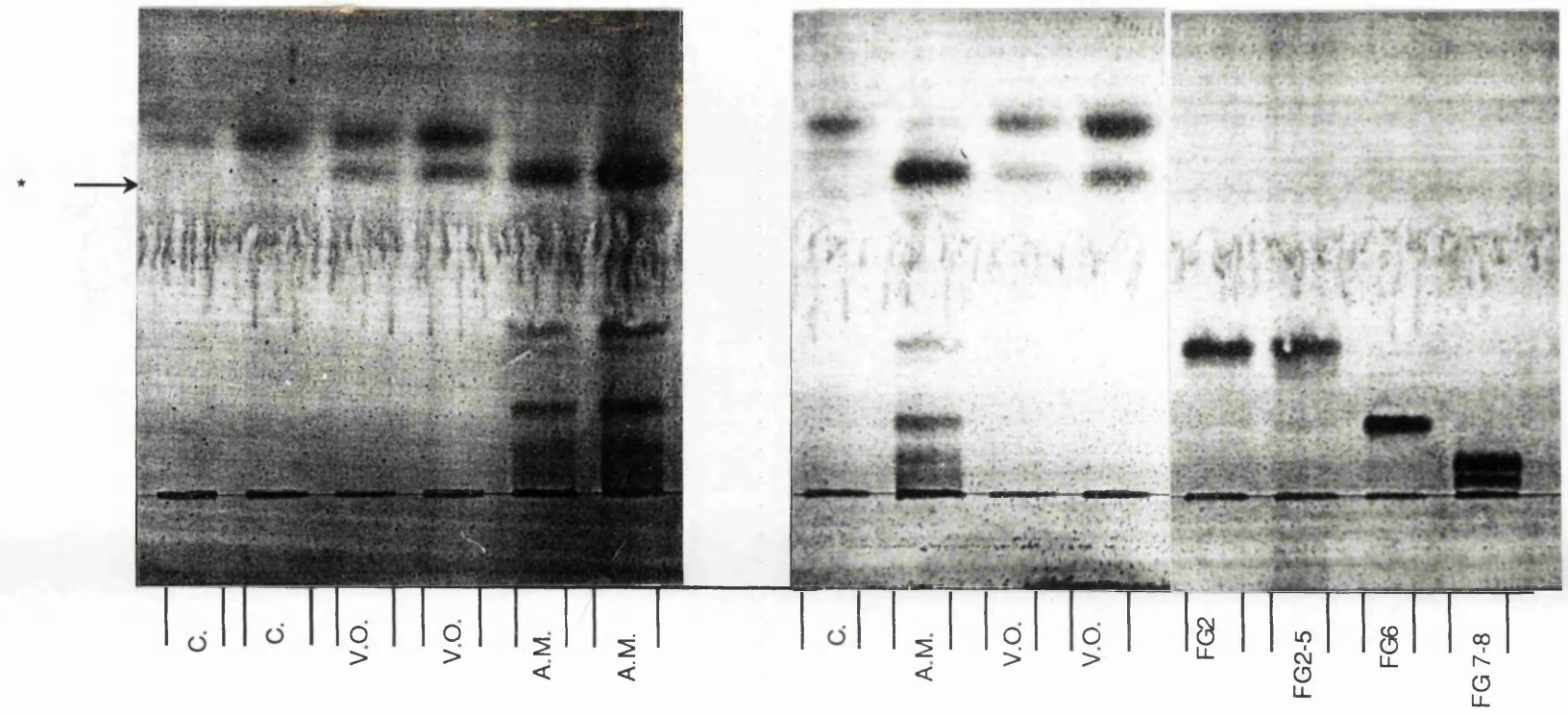


Fig 3.5a Thin layer chromatography of urines.

C, control.

FG2, FG2-5, FG6, FG7-8; markers.

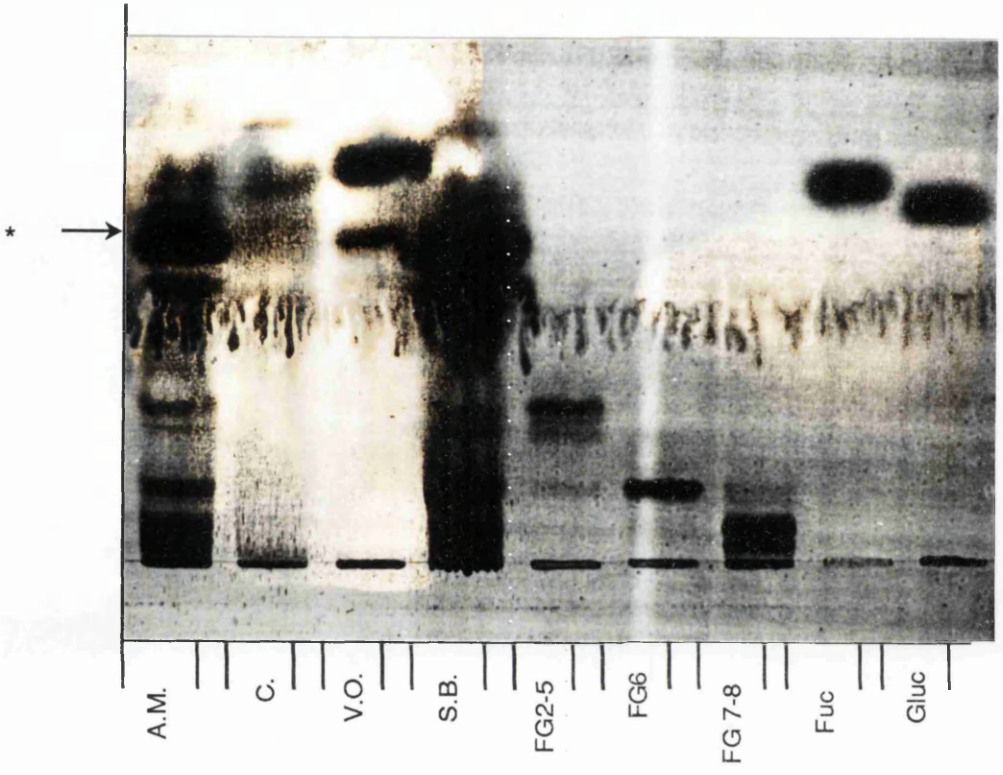


Fig 3.5b Thin layer chromatography of urines.

C, control.

FG2, FG2-5, FG6, FG7-8; markers.

Fuc, fucose.

concentration of urine was loaded. A low molecular weight band (marked with an asterisk) was present in the urine of patient V.O., but not in control urine. This low molecular weight band was also present in the urine from the two typical fucosidosis patients A.M. and S.B.

In summary, thin layer chromatography of urine from S.B. and A.M. revealed the presence of high molecular weight storage products typical of fucosidosis patients, whereas that of V.O. showed an atypical pattern.

3.5.Cross reacting immunological material (CRIM)

3.5a.Quantitative measurement of α-L-fucosidase protein by a double antibody sandwich enzyme linked immunoabsorbent assay (ELISA).

A two antibody sandwich ELISA was developed to estimate the amount of fucosidase protein expressed in fibroblast or lymphoblastoid cell lines from the fucosidosis patients (fig 3.6).

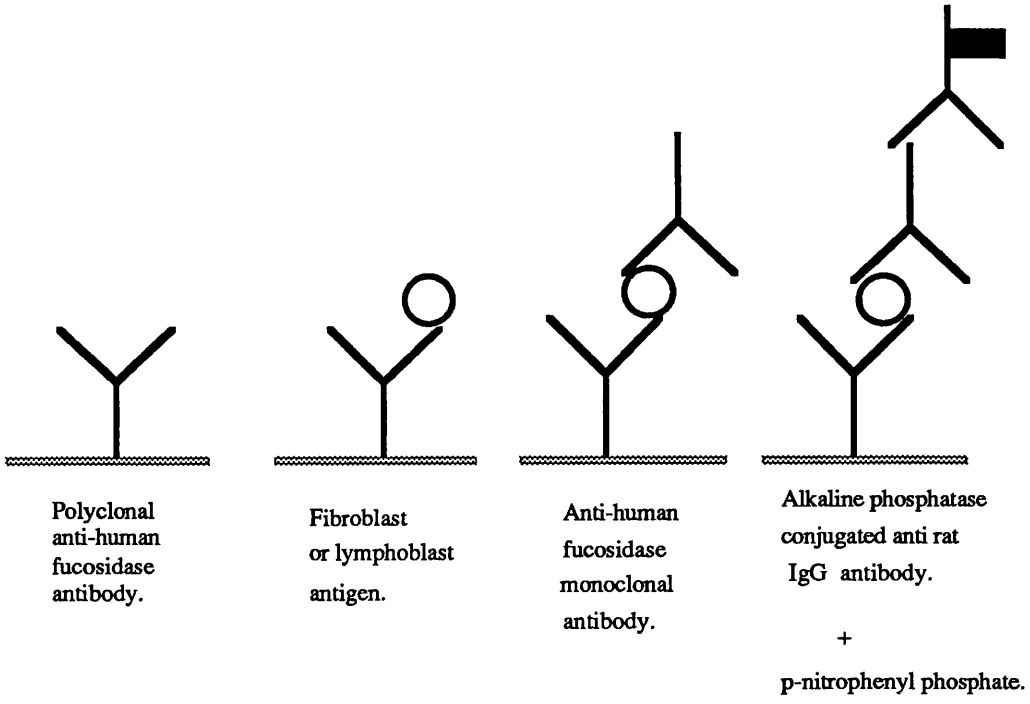


Fig. 3.6. Diagram to show the technique of two antibody sandwich ELISA for the detection of fucosidase antigen.

1υg of polyclonal anti-human fucosidase serum was used to coat each well of a microtitre plate. This is greater than the binding capacity of the well. Following a wash to remove excess first antibody, the remaining sites for protein binding on the PVC plate were blocked by incubation with BSA in a buffer. Following a futher wash 20υg/ml, 40υg/ml or 80υg/ml of crude fibroblast or lymphoblast extract was added as antigen. The appropriate amount of rat anti human fucosidase monoclonal IgG to be used as second antibody was determined by using increasing dilutions in preliminary experiments. Dilutions of 1:400 and above gave a higher background of nonspecific binding. A dilution of 1:800 was therefore

chosen. The dilution of alkaline phosphatase conjugated goat anti-rat IgG antibody to be used was ascertained by varying the dilution of conjugate. A dilution of 1:500 was chosen because it was the lowest amount to give high sensitivity. For each assay negative controls of no first antibody, no second antibody, no third antibody or no antigen, were carried out. Control fibroblast or lymphoblast extracts were assayed alongside the fibroblasts extracts of the fucosidosis patients for each experiment. The average background absorbance of the no antigen controls were subtracted from each reading, and the results were expressed as a percentage of the absorbance of control fibroblasts or lymphoblasts. Each assay was repeated at least once, and the results were consistent. The results presented are the average values of separate experiments for each fibroblast or lymphoblast type (fig 3.7 - 3.10).

For a given primary antibody concentration the number of antigen molecules bound will be proportional to the antigen concentration until the antibody binding sites are saturated. An almost linear relationship between CRIM and total protein concentration is seen for the control fibroblasts up to 40υg/ml (fig 3.11). At a total protein concentration of above 40υg/ml the antibody binding sites appear to become saturated in the case of controls 3 and 4. CRIM values are therefore presented as a percentage of controls at a total protein concentration of 40υg/ml.

Patient		**%CRIM** (at 40υg/ml total protein)
A.M.		6.9%
G.J.		7.5%
C.L.		3.6%
C.B.		36.2%
V.O.	fibroblasts	117.8%
	lymphoblasts	90.0%

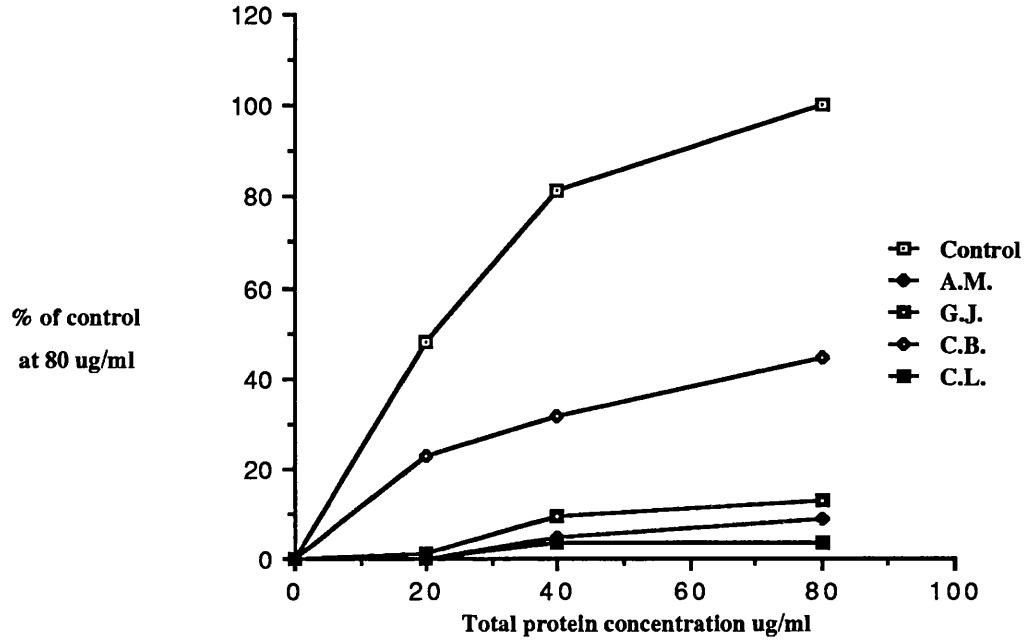


Fig.3.7. CRIM values expressed as a % of control fibroblasts.

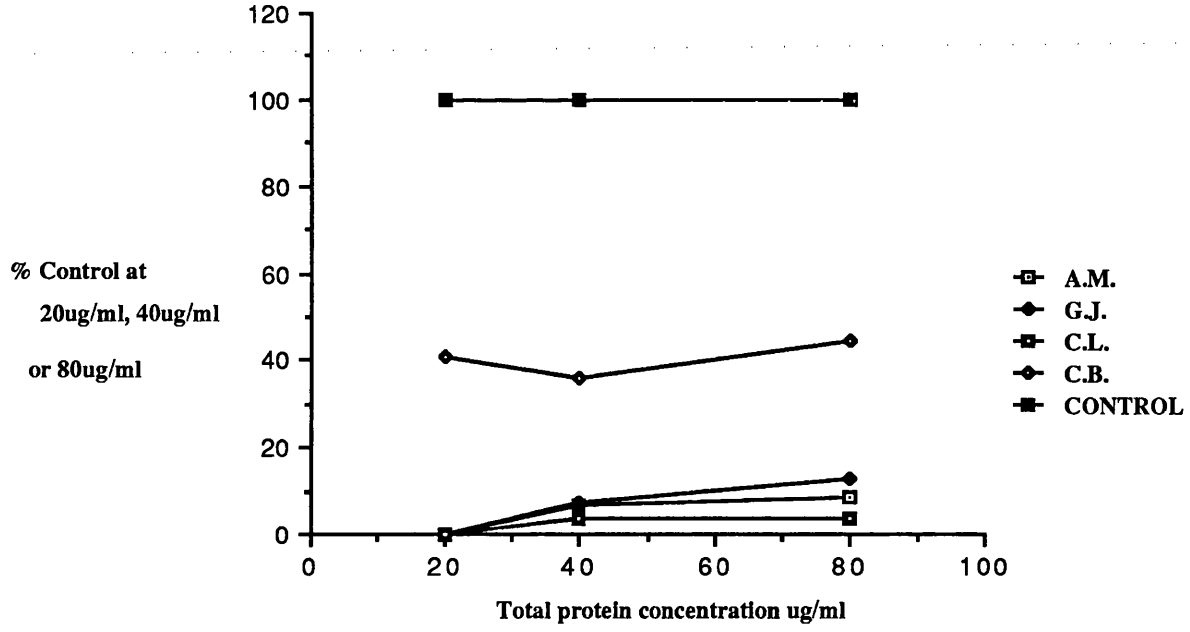


Fig.3.8. % CRIM at different total protein concentrations.

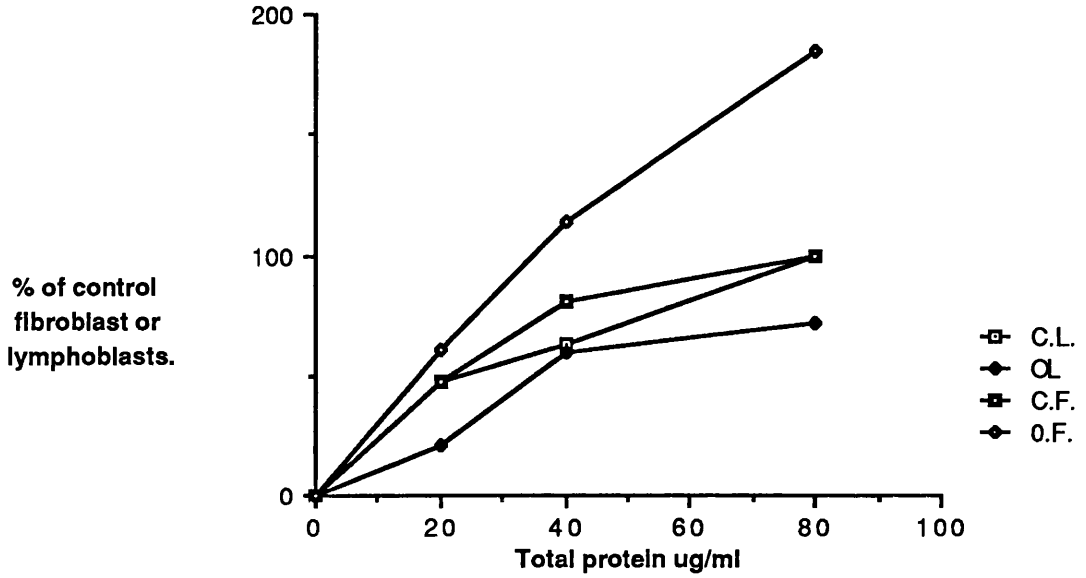


Fig.3.9. Fibroblast and lymphoblast CRIM in controls and patient V.O.

C.L.,control lymphoblasts; C.F.control fibroblasts; O.L.,V.O. lymphoblasts; O.F., V.O.fibroblasts.

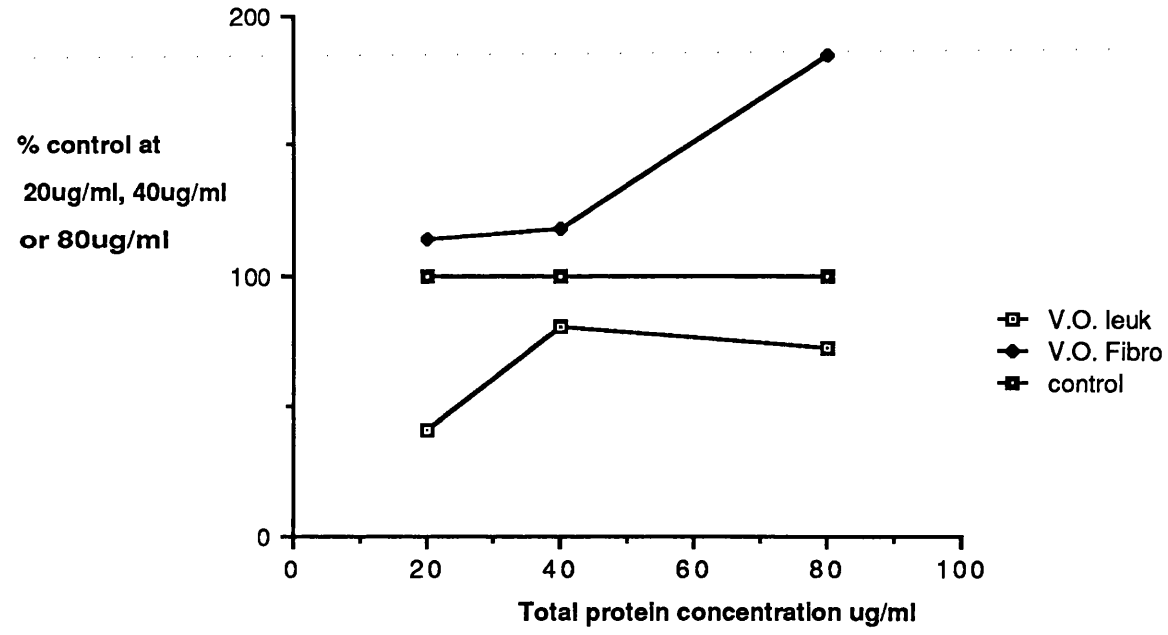


Fig.3.10. % CRIM in patient V.O. lymphoblasts and fibroblasts at different total protein concentrations.

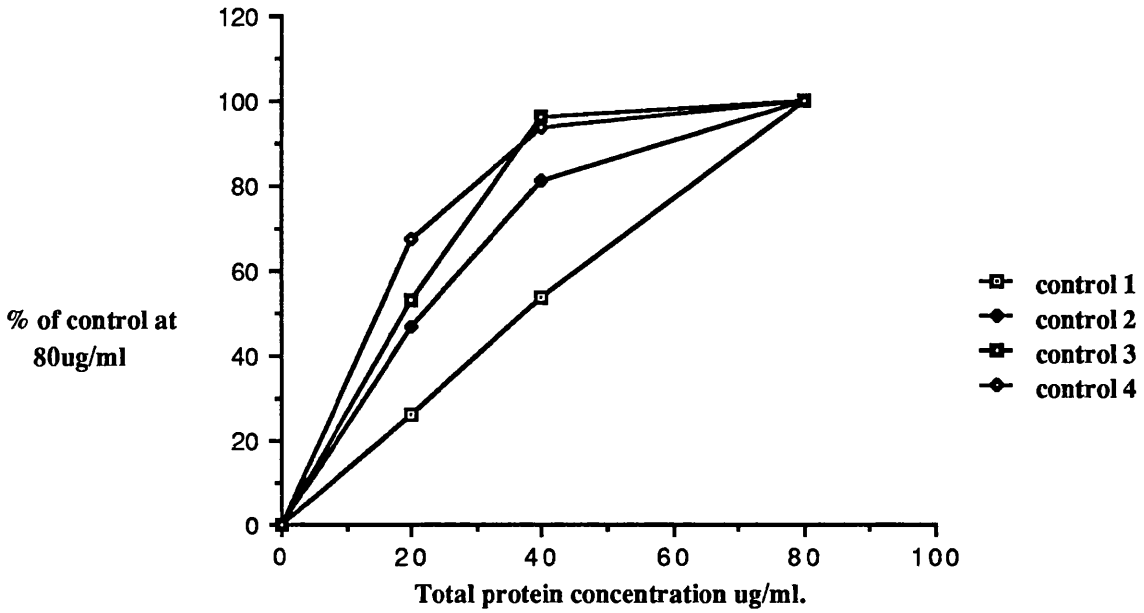


Fig.3.11. Comparison of CRIM values at different total protein concentrations for 4 different control fibroblasts.

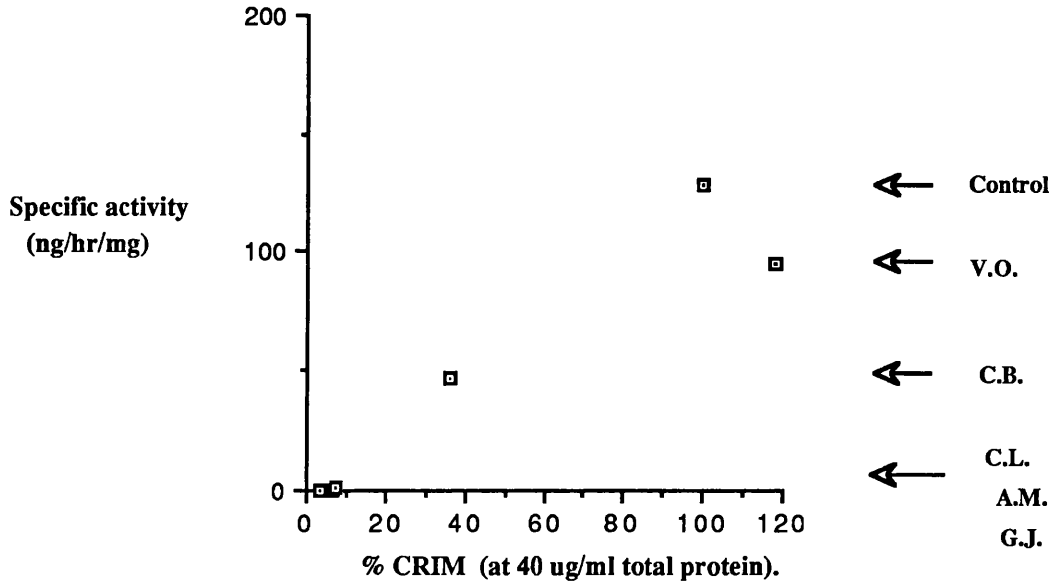


Fig.3.12. The relationship between specific activity and CRIM in fibroblasts.

Patients A.M., C.L. and G.J. had negligible anti-fucosidase CRIM. These values are typical for fucosidosis patients. Willems et al. (1991) found residual fucosidase protein to be below 6% of the normal mean in lymphoblasts of 23 fucosidosis patients. A.M., C.L. and G.J. also had negligible enzyme activity (fig 3.12).

Patient C.B. had intermediate levels of CRIM (36.2% of control fibroblasts). His enzyme activity was also intermediate (fig 3.12). This suggests a reduced amount of enzyme of normal activity is being produced.

Fibroblasts from patient V.O. (taken at 2 years of age) had a higher level of anti-fucosidase CRIM than fibroblast controls (117.8% of control fibroblasts). Fibroblast enzyme activity is reduced in this patient. This is consistent with the production of increased amounts of enzyme with a decreased activity (fig 3.12). The ratio of % CRIM to % activity of lymphoblasts (90% and 89% of controls respectively) taken at ten years of age was approximately the same as controls for patient V.O., however.

This assay was used to detect the presence of residual fucosidase protein expressed in cell extracts of fucosidosis patients. However, the putative disease causing mutation may produce fucosidase with epitopes no longer recognised by the monoclonal or polyclonal antibodies. This would lead to false negative results.

3.6 Discussion

Of the six fucosidosis patients in this study, four had a phenotype at the protein level which is typical of fucosidosis patients. All four patients S.B., G.J., C.L. and A.M. had fucosidase activity in fibroblasts or leukocytes of less than 1% of control levels. G.J., C.L., and A.M. also had negligible anti-fucosidase CRIM. A typical pattern of high molecular weight storage products was detected in the urine of S.B. and A.M.

All four of these patients would be classified as having type 2 fucosidosis. The

patients A.M., S.B., and C.L. all had undetectable leukocyte fucosidase activity and yet had differing severities of phenotype (Table 3.5). A.M had an earlier onset of initial symptoms than C.L. and a more rapid neurological and mental deterioration. A.M., for example, lost the ability to sit unsupported at 4-6 y whereas C.L. retained the ability to do so until over 20 yr of age.

The parents of S.B. and A.M. had intermediate levels of fucosidase activity as expected for obligate heterozygotes. Assignment of carriers of fucosidosis among the siblings of S.B. using enzyme assays was ambiguous, especially in the case of Sa.B.(Table 3.2).

The patient, C.B., had intermediate levels of fibroblast activity and anti-fucosidase CRIM, suggesting that a reduced amount of fucosidase of normal specific activity is produced. Van Elsen et al. (1983) found that phenotypically normal individuals that are homozygous for the low plasma fucosidase activity polymorphism allele also have a reduced fibroblast fucosidase activity, less than 50% of controls. Homozygotes for the low activity polymorphism have reduced amounts of enzyme of the same specific activity as controls (Dioccio et al. 1986), as these results show for the patient C.B. The patient C.B. may therefore be a variant case of fucosidosis with high residual activity, or may have another neurological disorder and be homozygous for the low activity polymorphism. Leukocyte activity would determine which is the case.

The patient V.O. is atypical of fucosidosis patients. At the age of two years her leukocyte and to a lesser extent, fibroblast fucosidase activities were low (25.3%, 8.1% and 35.3% of the control mean respectively) but above those typically found in fucosidosis patients. At ten years of age, her leukocyte activity had increased to control levels. The reduced leukocyte activity at two years of age cannot be due to the low plasma fucosidase activity polymorphism since the polymorphism is not expressed to a significant extent in leukocytes. The residual fibroblast activity had a slighty altered pH-profile, with a shift to less acidic pH, which is consistent with a relative increase in the monomeric form of fucosidase, type 2. This may reflect a reduced tendency of the subunits to associate. The very low level of

residual activity purified from the liver of a fucosidosis patient by Alhadeff and Andrews-Smith (1980b) had a pH-activity profile similar to controls above pH 4.5. No activity was detected below pH 4.5 in the patient reported by Alhadeff, whereas controls had 50% maximum activity below pH 4.5. The Km of the fucosidase from fibroblasts of patient V.O. was similar to controls. In contrast to these results an increase in Km has been found for fucosidase extracted from other fucosidosis patients. Alhadeff and Andrews-Smith, (1980b) found a 4-5 fold increase in the value of Km above controls in the enzyme extracted from the liver of a fucosidosis patient. Troost et al. (1976) found a similar increase in the value of Km in leukocytes and sera from a patient. The enzyme from patient V.O. was more thermostable than the control enzyme. In contrast fucosidase isolated from liver of one patient or leukocytes of another patient showed a marked decrease in thermostability (Alhadeff and Andrews-Smith, 1980b; Dimateo et al. 1976). Immunoprecipitation experiments demonstrated that the fucosidase enzyme extracted from the fibroblasts of V.O. is recognised by the anti-fucosidase antisera used. Therefore any putative mutation in the fucosidase gene of this patient does not change epitopes in such a way that they are no longer recognised by the antibodies. Results from the two antibody ELISA experiments suggest that the patient V.O. expressed an increased quantity of fucosidase of reduced specific activity in fibroblasts at two years of age. By ten years of age lymphoblastoid cells from this patient had fucosidase CRIM and activity within the normal range, however.

A similar atypical fucosidosis patient has not so far been reported.

Comparison of the clinical details of the fucosidosis patients.

	A.M	S.B.	C.L.	V.O.
Mental retardation	yes	yes	yes	moderate
Neurological deterioration	yes	yes	yes	
Coarse facies	yes	yes	yes	moderate
Kyphoscolosis	yes	yes	no	
Angiokeratoma	yes	no	yes	no
Joint contractures	yes	yes	moderate	
Seizures	yes			
Visceromegaly		no	no	
Age of initial symptoms.	~4yr	~18months	Angiokeratoma~5yr	
Age at which able to sit	6 months	8 months	normal age	normal age
Age at which lost ability to sit.	4-6 yr	Retained ability at 8 yr	Retained ability at 38 yr.	Retained ability
Age at which able to walk.	2 yr	16 months	normal age.	normal age
Age at which lost ability to walk.	4 yr	Deterioration in ability at 6 yr	Deterioration in ability noted at 11 yr.	able to walk at 11yr.
Age still living.	15 yr	8 yr	38 yr	11yr.
Consanguinuity	Not reported.	yes	yes	Not reported.
Fucosidase activity: (ng/hr/mg)				
leukocytes	0	0	0	2yr: 6.5, 11yr: 146
fibroblasts	0.46		0.42	2yr: 44

Table 3.5

CHAPTER 4.

Fucosidase mRNA expression.

4.1. Introduction.

Insight can be gained into the defect underlying fucosidosis and other lysosomal storage diseases by studying the expression of mRNA. CRIM negative patients may have a complete absence of mRNA either due to transcript instability or lack of mRNA expression, for example due to a defect in the promoter. CRIM positive patients may produce specific transcripts of abnormal size or quantity. Transcripts of abnormal size may be a result of abnormal splicing, possibly due to a point mutation creating or destroying a splice site. Large insertions or deletions would also give rise to transcripts of abnormal size. Expression of fucosidase mRNA in patients and controls was therefore investigated.

4.2. Results.

4.2.a Northern blotting.

Total RNA was extracted from fibroblasts or lymphoblasts of patients and controls. The RNA was separated by electrophoresis in an agarose gel (1.2%, 0.6M formaldehyde). Inclusion of ethidium bromide in the sample buffer allowed the RNA to be visualised directly and photographed under UV. The RNA samples could thereby be checked for degradation (fig 4.1). The RNA was transferred to a nylon filter by Northern blotting and was hybridized to fucosidase cDNA probe A+B. The position of the bands on the autoradiogram could be related to those seen on the agarose gel by measuring their distance of migration from the origin. A band representing hybridization to 28S ribosomal RNA (rRNA) could be seen in each lane on the autoradiogram (fig 4.2). No bands representing hybridization to the 18S rRNA were present however. A band of 2.5kb could be seen in RNA extracted from lymphoblasts of the control and patient V.O. This probably represents fucosidase mRNA. These results show that the fucosidase lymphoblast mRNA from patient V.O. is the same size and has approximately the same level of expression as control lymphoblast mRNA.

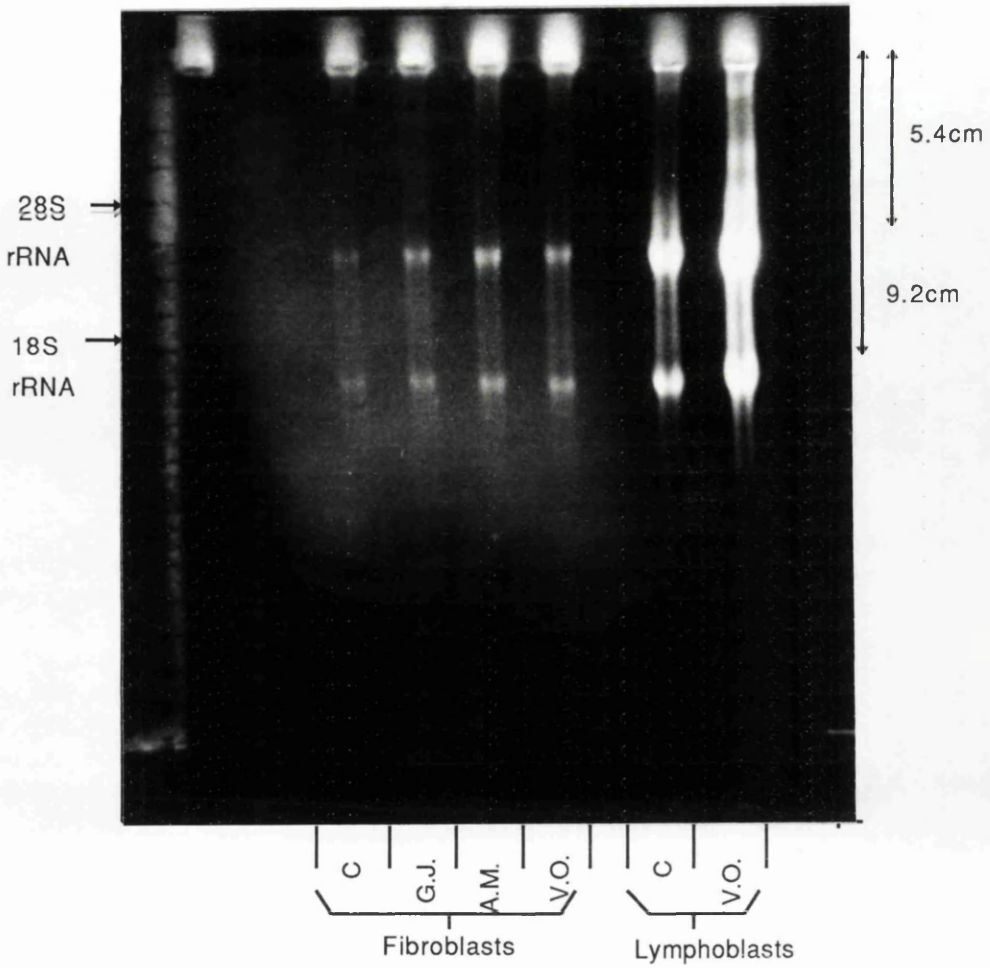


Fig 4.1. Agarose gel of RNA samples photographed under uv. Ethidium bromide was included in the sample buffer.

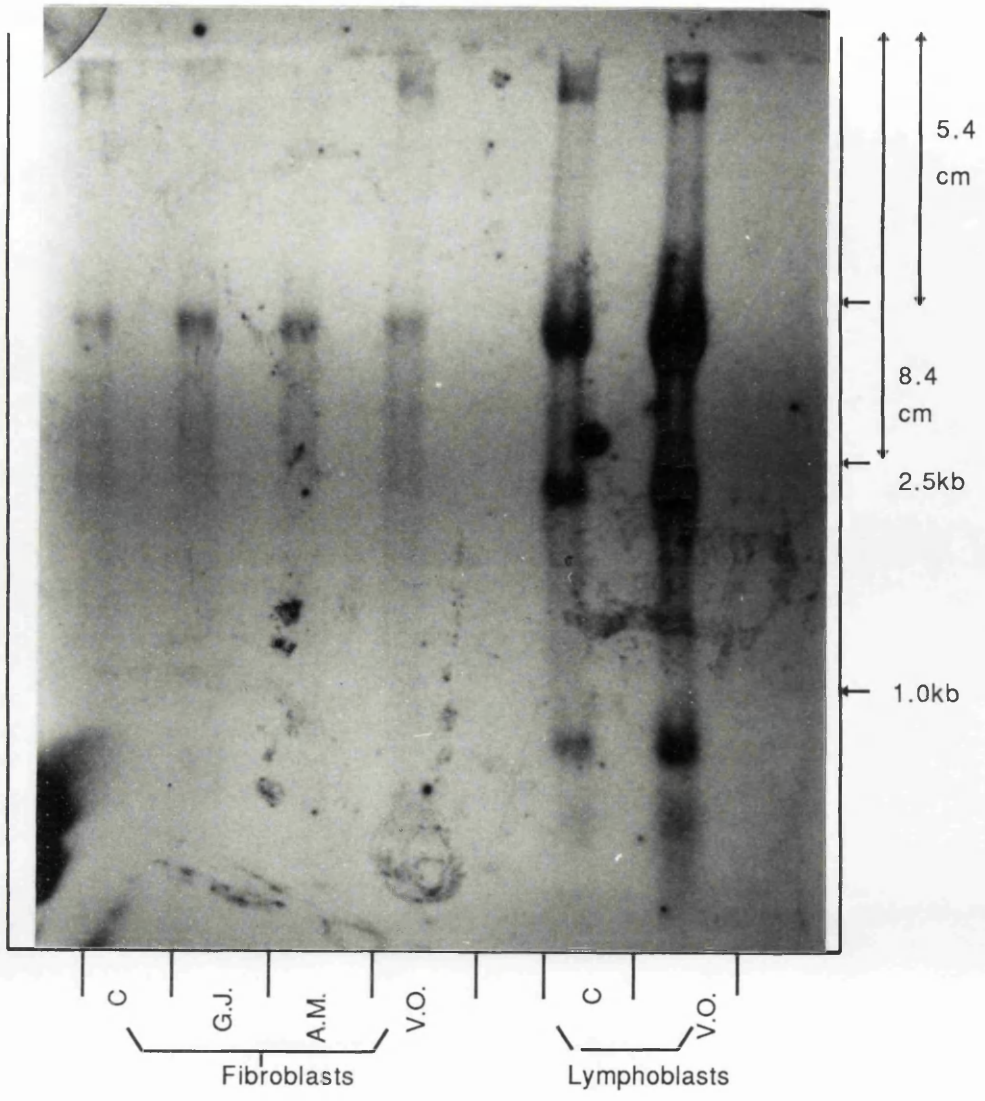


Fig 4.2. Northern blot of the agarose gel in fig 4.1 hybridised to fucosidase cDNA probe A+B.

A very faint band representing fucosidase mRNA was visible on the autoradiogram of control fibroblast RNA, but not fibroblast RNA from patients A.M., G.J., or V.O. The absence of a 2.5kb band in the patient fibroblast RNA is probably not significant because that from the control fibroblasts is so faint. Fucosidase mRNA is expressed in control cells at a very low level. Fucosidase represents approximately 0.01% of total protein, as determined by the screening of a cDNA library and comparing the activity of pure enzyme with its activity in liver (Fukushima et al., 1985). The amount of fucosidase mRNA loaded in 20ug of total RNA may have been to low to be detected by this method.

4.2b. Amplification of cDNA.

Total RNA from controls and patients was reverse transcribed using random hexanucleotide primers and then amplified using fucosidase cDNA specific primers. The aim was to establish whether fucosidase mRNA was expressed in the patients and to determine whether any transcript produced was of normal size. The primers used for the PCR were complementary to a part of exon I and exon 8 of the cDNA, to produce a PCR product of 1169bp.

Several bands were seen on the ethidium bromide stained gel of PCR products following amplification with an annealing temperature of 56^{o}C. Some of these may represent non specific priming. The annealing temperature was consequently increased to 60^{o}C. Control fibroblasts had reverse transcribed and amplified cDNA products of approximately 1.2kb and 0.9 kb (fig 4.3). The upper band of approximately 1.2kb is about the size expected following amplification using these primers (the calculated length from the cDNA sequence was 1,169bp). This may therefore represent the normal fucosidase transcript. The smaller product of approximately 0.9kb may represent an alternative fucosidase transcript, as has been found in RNA processing of several other lysosomal enzymes. The smaller band may alternatively be a result of non specific priming from a sequence similar to those of the priming sites.

RNA from fibroblasts of patient V.O. had two bands of the same size as control

fibroblasts, indicating that mRNA of the same size and approximately the same quantity is expressed in fibroblasts of this patient.

RNA from patient G.J. had only the band of approximately 0.9kb possibly representing an alternative transcript. The cDNA of 1.2kb was absent.

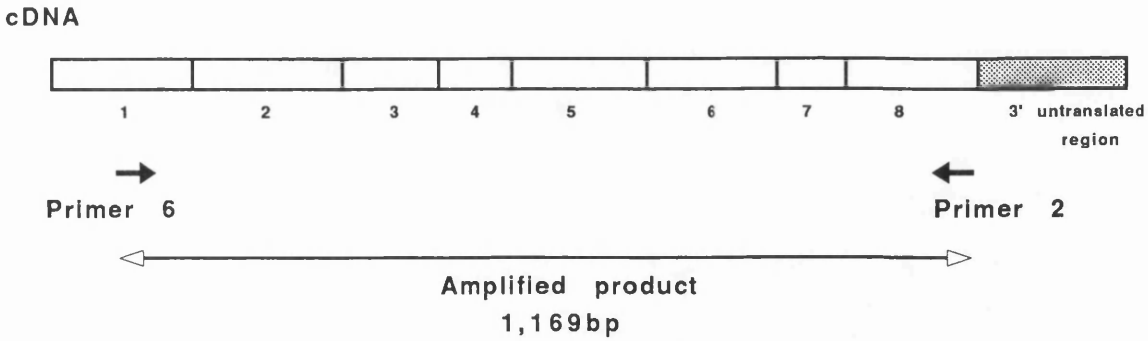
No amplified product was present after amplification of cDNA from patient A.M., suggesting that no fucosidase mRNA is produced in this patient.

Amplified cDNA from the lymphoblasts of V.O. had the 0.9kb band, also seen in the amplified products of fibroblasts of controls and patients, V.O.and G.J. The 1.2kb band was absent however and three novel bands were present, of approximately 1.4kb, 1.0kb and 0.8kb.

The primers used for amplification of the cDNA had some homology with the fucosidase pseudogene. The pseudogene has 13 out of 20 nucleotides in common with the 5' primer site of fucosidase cDNA, and 14 out of 20 nucleotides in common with the 3' primer site. Amplification of the pseudogene would give rise to 1,130bp product (which would probably not be resolved on the gels used from the 1,169bp cDNA product). If amplification of pseudogene sequences had occurred one would expect to see a band of 1,130bp in each lane, which was not the case. The primers would not amplify any contaminating genomic DNA as this would give rise to too large a product. The bands from the agarose minigel were excised and reamplified individually (fig 4.4). Attempts to sequence them directly were unsuccessful.

4.3. Discussion.

Northern blotting and hybridization showed that the lymphoblasts of patient V.O. had fucosidase mRNA of the same size (2.5kb) and quantity as control lymphoblasts. Fucosidase mRNA of 2.3kb has been reported by Guazzi et al., (1989). Small differences in transcript size would not be detected by this method.



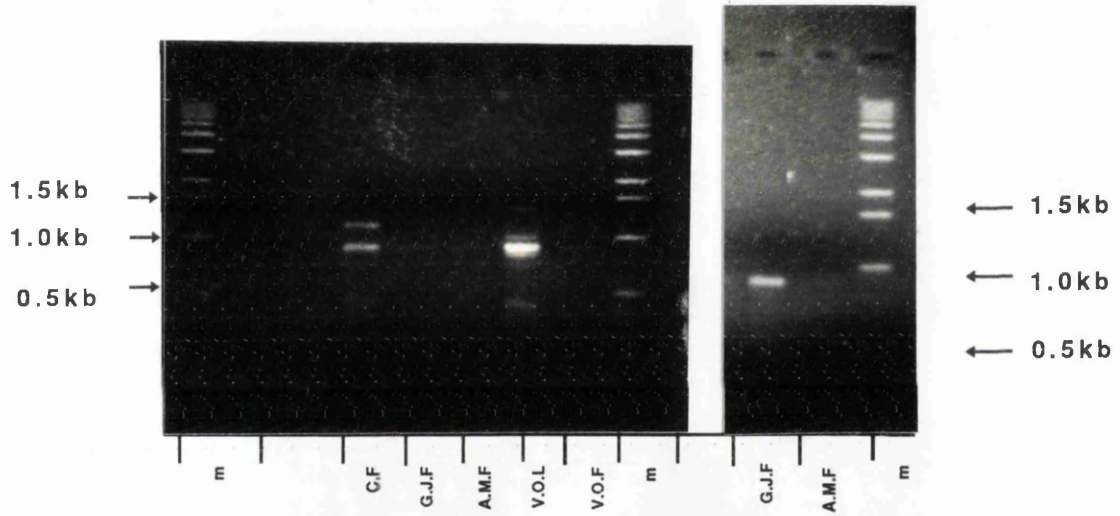


Fig 4.3 Reverse transcribed and amplified PCR products.

(60oC annealing temperature) C.F., control fibroblasts; G.J.F, fibroblasts from G.J. A.M.F, fibroblasts from A.M.; V.O.L, lymphoblasts from V.O. V.O.F, fibroblasts from V.O.

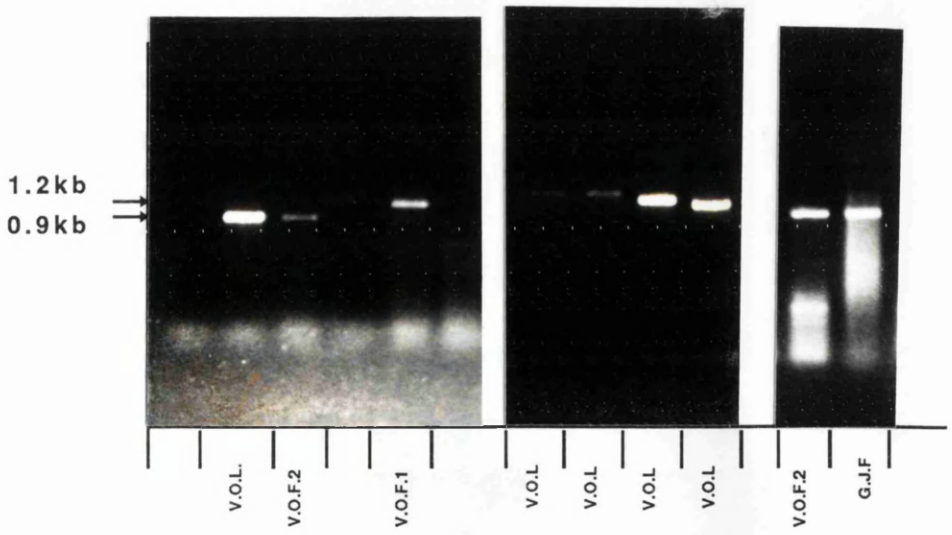


Fig 4.4 RTPCR products reamplified individually.

Amplification of reverse transcribed fucosidase mRNA indicated the presence of two fucosidase transcripts in controls. One amplified product of 1.2 kb was of the expected size as deduced from the published cDNA sequence. The second product was smaller than expected- approximately 0.9kb. The identity of these amplified products canot be unequivocally determined until the products are sequenced.

The smaller product may result from alternative splicing of the fucosidase mRNA. This has been reported for several other lysosomal enzymes. Three minor aspartylglycosaminidase homologous fragments were consistently amplified together with the major product following PCR of cDNA with aspartylglycosaminidase specific primers (Fisher et al., 1991). Two of these products were found to have the same sequence as the major aspartylglycosaminidase product, except that one had a 30bp deletion and the other a 76bp deletion, suggesting an alternative splicing mechanism. An alternative transcript, with 172bp of the internal sequence being replaced by a novel 40bp sequence, has been found in acid sphingomyelinase (ASM) RNA, accounting for 10% of the ASM transcripts (Quintern et al., 1989). Two types of β-glucuronidase cDNA have been identified one with a 153bp deletion within the coding sequence. Only the full length mRNA produced a protein with β-glucuronidase activity (Oshima et al., 1987). Similarly alternative splicing of β-galactosidase RNA results in a minor transcript lacking two non contiguous protein coding regions and producing a catalytically inactive protein (Morreau et al., 1989). Alternative transcripts have also been found for α-Nacetyl galactosaminidase (Yamauchi et al., 1990). The function of the alternative transcripts, if any, is unclear.

Both the normal sized and the smaller transcript appear to be expressed in control fibroblasts and fibroblasts from patient V.O. Only the smaller transcript appears to be present in RNA from fibroblasts of patient G.J. The normal sized transcript was absent. Similarly one aspartylglucosaminuria patient was found to have increased amounts of the two minor transcripts relative to the major transcript (Fisher et al., 1991).

Neither cDNA band was present after amplification of cDNA from the fibroblasts of

patient A.M., suggesting that no fucosidase mRNA is produced in this patient.

cDNA from lymphoblasts of patient V.O. lacked the transcript of normal size but had increased levels of the apparently alternatively spliced transcript. Three other amplified cDNA bands were also present of approximately 1.4kb, 1.0kb and 0.8kb. This may represent different processing of fucosidase mRNA transcripts between fibroblasts and lymphoblasts of patient V.O. At the age of two years this patient had intermediate fibroblast activity and very low leukocyte activity. This may on the other hand represent a normal difference in RNA processing between tissue types.

Guazzi et al (1989) investigated mRNA production in 6 other fucosidosis patients by Northern blotting. Smaller differences in transcript size, for example produced by alternative splicing would not be detected by this method. Three of the patients produced mRNA of normal size and amount as appears to be the case for fibroblasts of patient V.O. No fucosidase mRNA was produced in two of the patients, as for patient A.M., and a decreased amount was produced in another patient.

CHAPTER 5.

Detection of previously identified mutations and polymorphisms in fucosidosis patients.

5.1. Introduction.

The six fucosidosis patients were next investigated at the level of the fucosidase gene with the aim of detecting disease- causing alterations in the DNA. Only two mutations causing fucosidosis have been found to date - a large deletion at the 3' end of the gene (Willems et al., in press) and a C-T transition generating a stop codon in exon 8 (Willems et al., 1988b). DNA from the six patients in the study was investigated to determine whether either of the two previously described mutations was present. Two restriction fragment length polymorphisms have been identified in the fucosidase gene which have a potential use in prenatal diagnosis and carrier detection of fucosidosis in informative families. Pvu 11 and Bgl identify 2 allele polymorphisms with bands of 7 or 6kb, and 12 or 6.5+5.5kb respectively. The patients and their families were haplotyped for these restriction fragment length polymorphisms with the aim of determining whether they were informative for linkage.

Results.

5.2. Previously identified mutations in fucosidosis.

5.2a.Fucosidase gene deletion.

DNA was extracted from fibroblasts, lymphoblastoid cell lines or the blood of fucosidosis patients, heterozygotes and controls. The DNA was digested with restriction endonucleases, subjected to agarose gel electrophoresis and transferred to nitrocellulose membranes according to the method of Southern (1975). The Southern blots were hybridized to ^{32} P-labelled fucosidase cDNA probes.

No large insertions, deletions or gene rearrangements were detected on Southern blots

of any of the six patients (although some heterozygous deletions would not be detected by these methods). The 3' deletion described by Willems et al. (in press) in two fucosidosis siblings was not found. This deletion mutation generates an abnormal pattern of bands following hybridization of probe B to Southern blots digested with EcoR1. The normal 4.5kb band is absent and a novel 12kb junction fragment is observed. Heterozygotes for this mutation would therefore be detected by the presence of a 12kb band on such Southern blots. A 12kb band was not present on Southern blots digested with *Eco*R I and hybridized to probe B in any of the patients (fig. 5.2), showing that none of the patients in this study are either homozygous or heterozygous for the mutation described by Willems et al., (in press). Fucosidosis is therefore probably a result of point mutations, small deletions or small insertions in these patients. These would not be detected on Southern blots unless a restriction site was created or destroyed.

5.2b. The mutation causing obliteration of an EcoR1 site in exon 8.

Willems et al. (1988b) have recently characterised a nonsense mutation in seven fucosidosis patients which obliterates an EcoR1 site in exon 8. In control DNA this site lies between a 4.5kb and a 1.5kb EcoR1 fragment. Obliteration of this site therefore results in the generation of a 6kb band and disappearance of a 4.5kb band on EcoR1 Southern blots hybridised to probe B. The presence of a 6kb band and absence of a 4.5kb band on EcoR1 digested Southerns hybridized to probe B is therefore diagnostic for this mutation (fig 5.1). With probe C, a 1.5kb band is seen in control DNA and a 6.0kb band in DNA with this mutation.

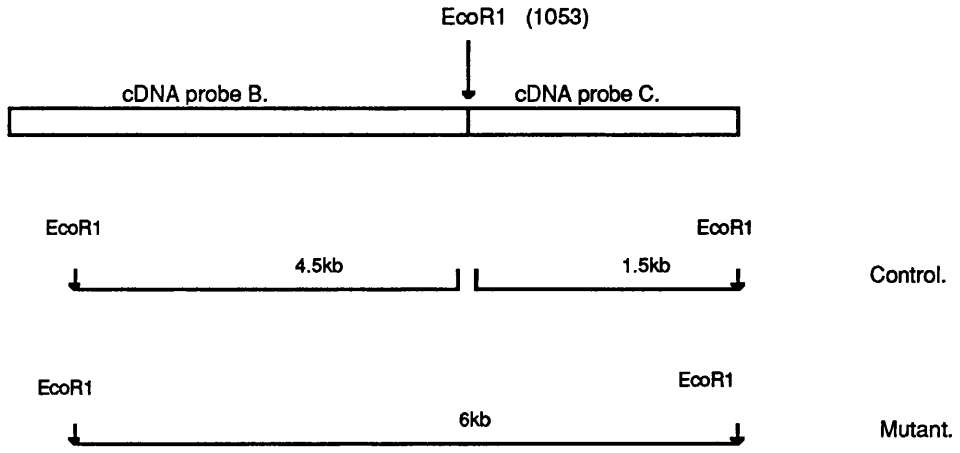


Fig. 5.1. Diagram to show the position of the EcoR1 (1053) site which is obliterated by a nonsense mutation in some fucosidosis patients (Willems et al. 1988b) in relation to fucosidase cDNA probes B and C.

To determine whether any of the patients in the study have this mutation in exon 8, Southern blots were made of EcoR1 digested DNA from each patient and controls. As can be seen from fig 5.2 a 4.5kb band was present on all the Southern blots after hybridization to probe B. None had a 6kb band with probe B or probe C (not shown). None of the patients therefore has the EcoR1 obliterated site mutation described by Willems et al. (1988b).

To confirm these results, the region close to and including exon 8 was amplified using the polymerase chain reaction (PCR). Primer 1 was constructed to be complementary to intronic sequences between exons 7 and 8 to ensure that no pseudogene sequences were amplified. Amplification using primers 1 and 2 produced a 257bp product which could be easily visualised on an agarose minigel stained with ethidium bromide. Digestion with EcoR1

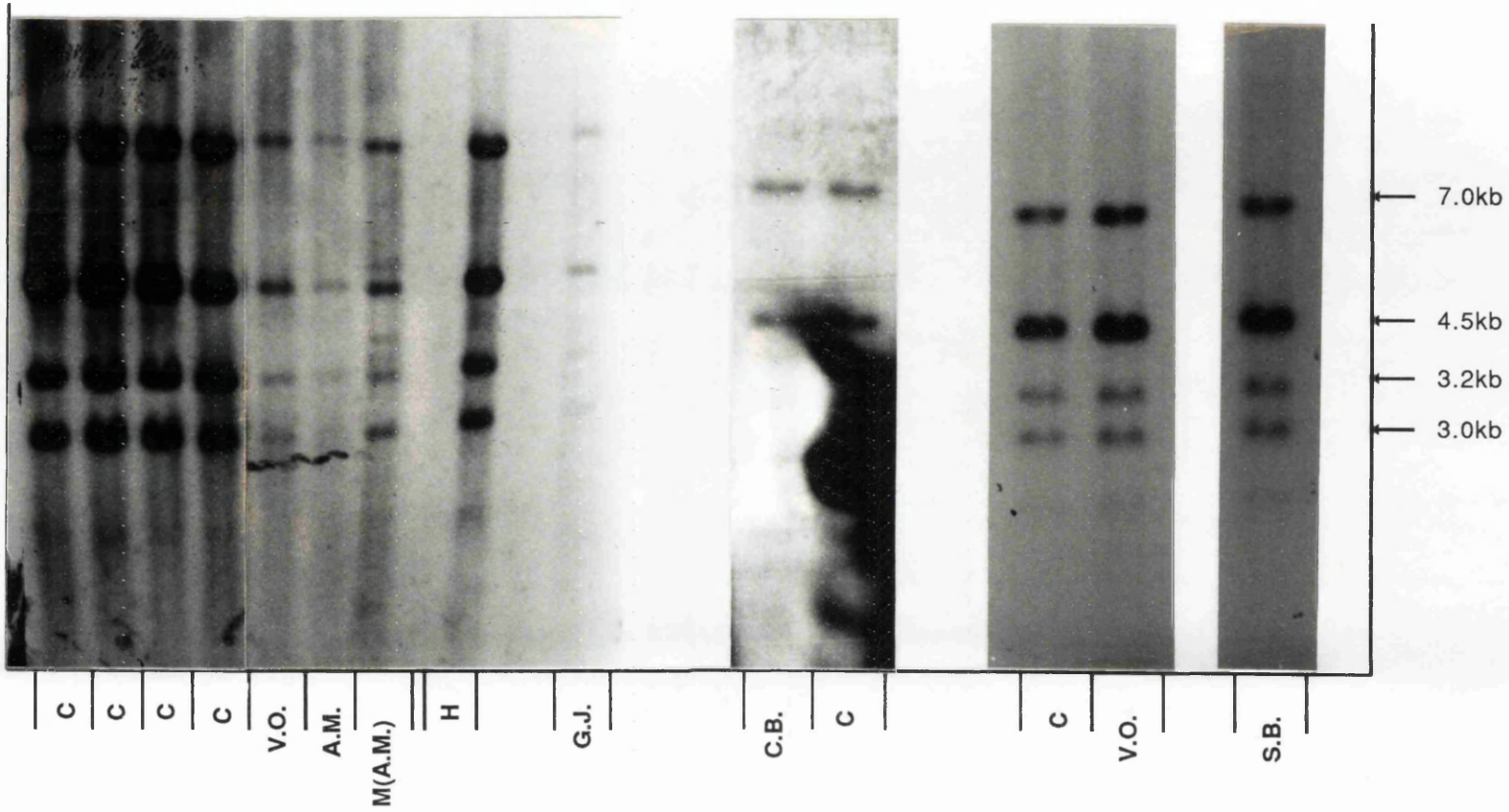


Fig. 5.2. Southern blots of DNA digested with EcoR1 and hybridised to fucosidase cDNA probe B. M(A.M.), mother of patient A.M. C, control DNA; H, DNA from a heterozygote for fucosidosis;

produced two fragments, one of 166bp and one of 91bp in all the patients and controls, showing that the EcoR1 site was intact(fig 5.3). There must consequently be other mutation(s) causing fucosidosis in these patients.

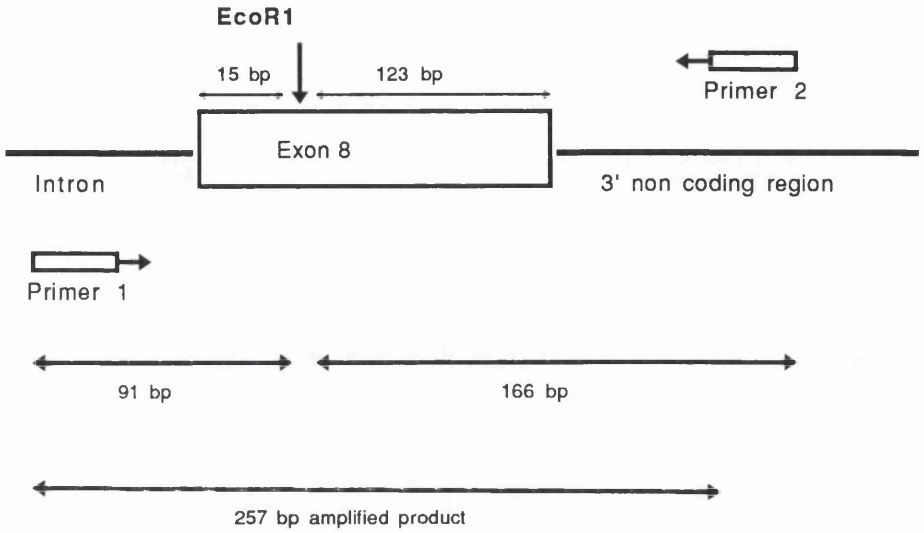
5.3.Restriction fragment length polymorphisms.

The Pvu11 and Bgl1 polymorphisms are tightly linked to the FUCA1 gene and can therefore be used in prenatal diagnosis if the family is informative for linkage. DNA was available from the parents of V.O. and S.B., and a limited amount from those of A.M. Alleles with the 7kb Pvu11 and the 12kb Bgl1 fragments are of haplotype 1, while alleles with the 6kb Pvu11 and the 6.5+5.5kb Bgl1 fragments are of haplotype 2 (as defined by Willems et al., in press). The restriction enzymes Bgl1 and Pvu11 were used to haplotype these patients and their families (fig 5.4 and 5.5).

As can be seen from fig 5.4 and 5.5, the disease segregates with the 6kb Pvu11 allele or 5.5+6.5 Bgl1 allele in the family of V.O. These RFLP's are therefore fully informative in this family. V.O. has the 2-2 haplotype (fig 5.6).

In the family of S.B. the disease segregates with the 7.0kb Pvu11 or 12kb Bgl1 allele from the father and one of the two 7.0kb Pvu11 or 12kb Bgl1 alleles from the mother. This family is therefore only partly informative. S.B. has the haplotype 1-1 (fig 5.6)

A.M. and his father and mother were all homozygous for the 7.0kb Pvu11 allele and the family is not therefore informative for linkage to the disease. A.M. was homozygous for the 5.5+6.5kb Bgl1 fragment as well as the 7kb Pvu 11 fragment. A.M. therefore has the haplotype 3-3 (fig5.6).



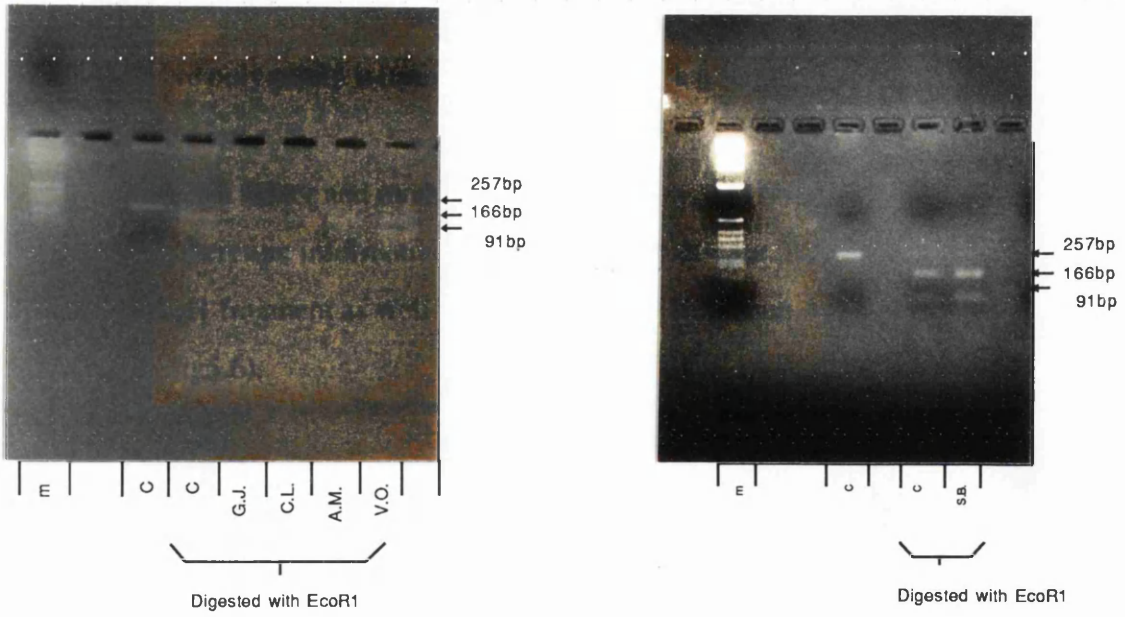


Fig 5.3 Amplified products of primers 1 and 2 digested with EcoR1.

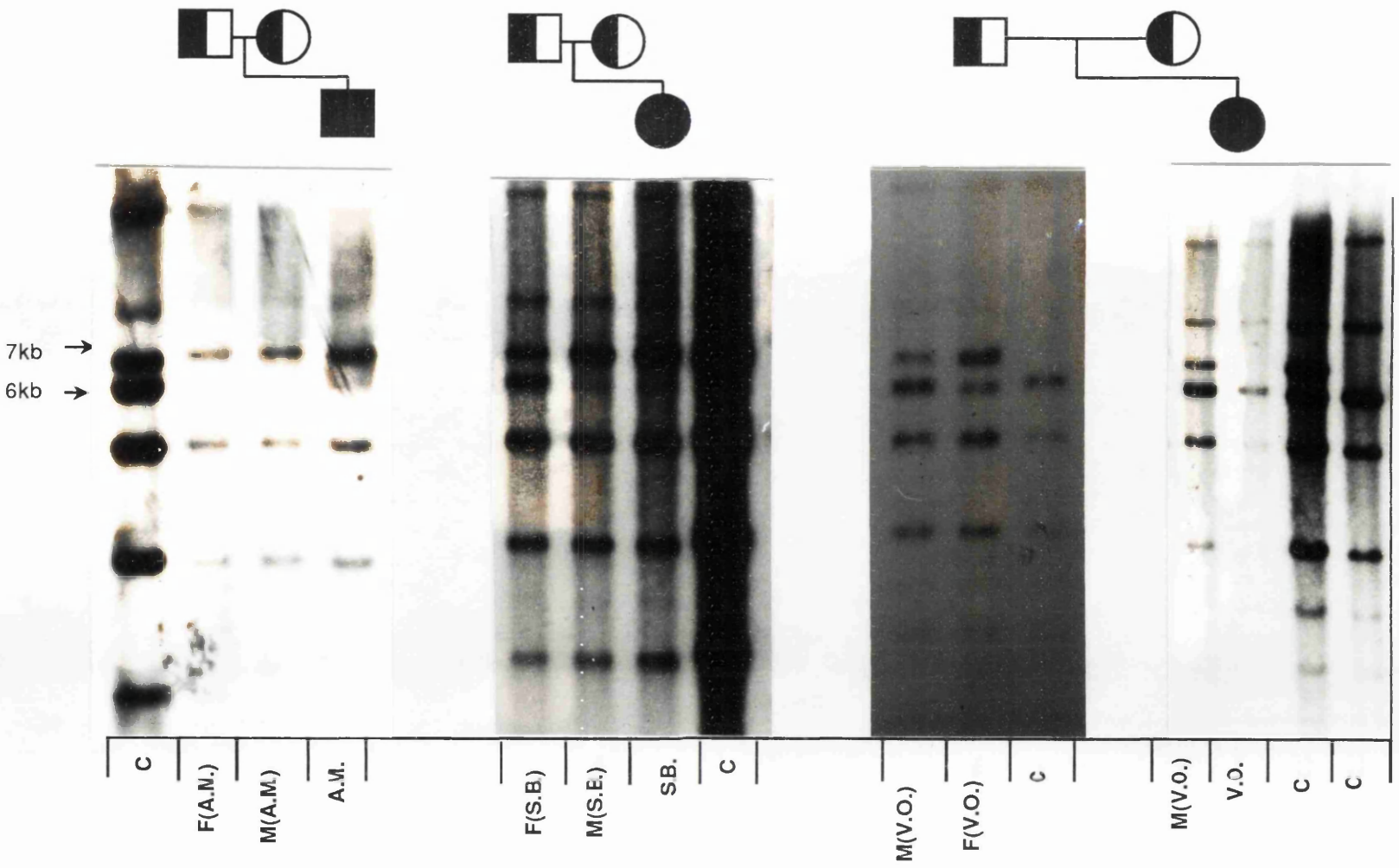


Fig. 5.4. Southern blots of DNA digested with Pvu11 and hybridised to fucosidase cDNA probe B.

C, control; M( ), mother of; F( ), father of.

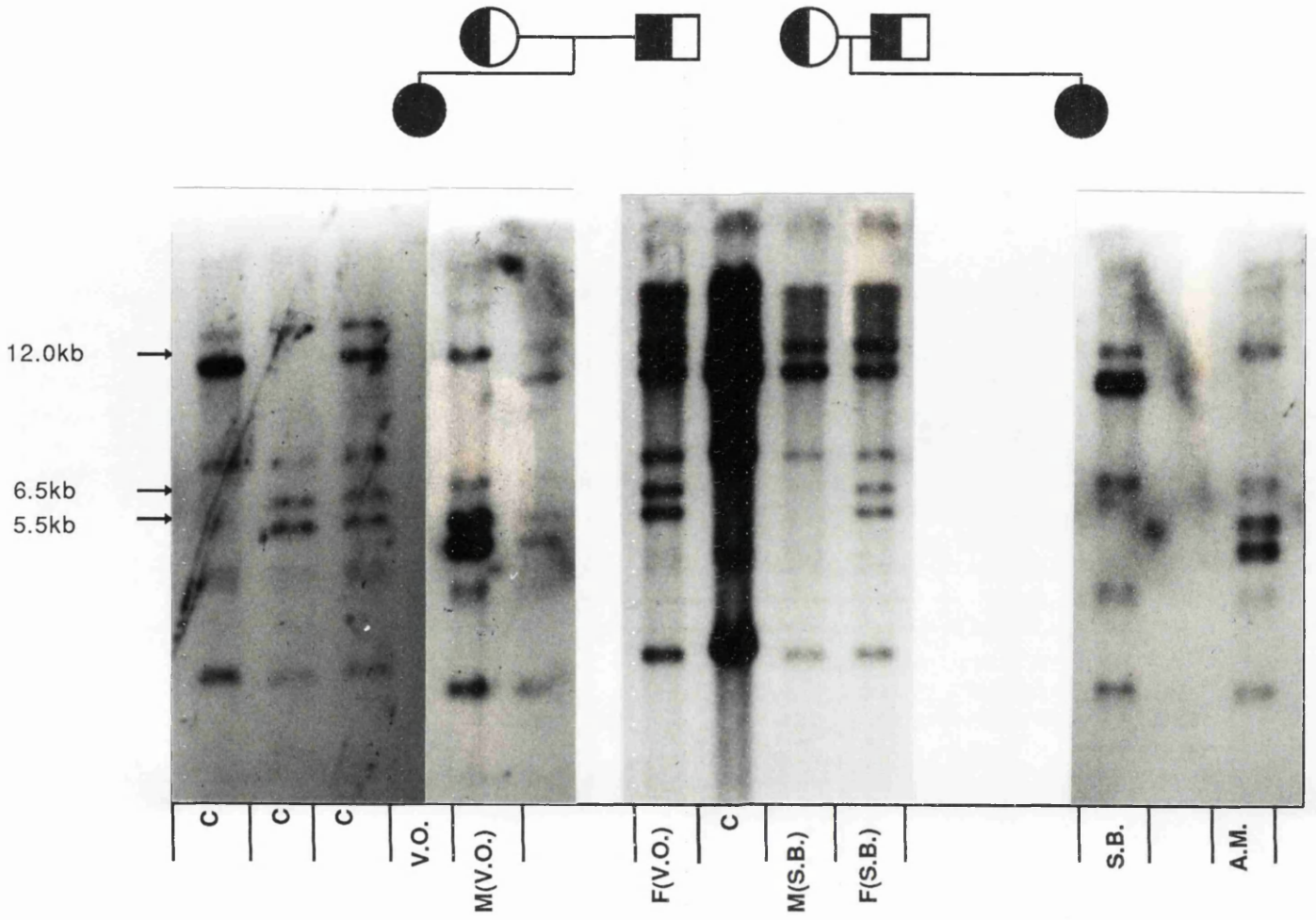
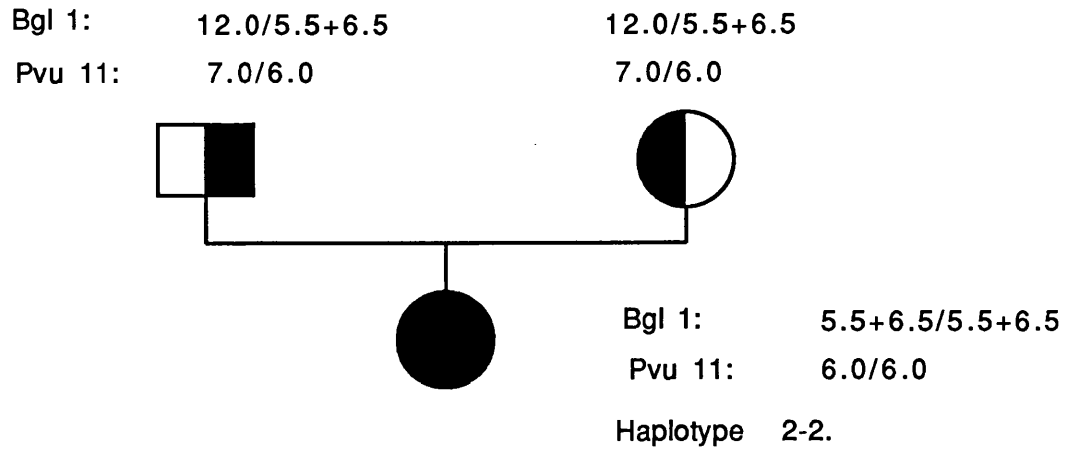


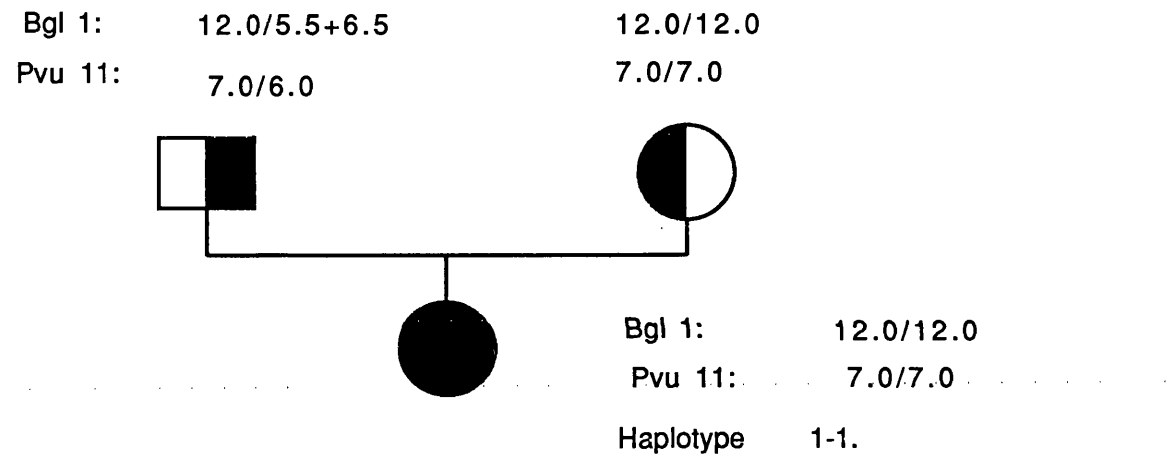
Fig. 5.5. Southern blots of DNA digested with Bgl1 and hybridised to fucosidase cDNA probe B.

M( ), mother of; F( ), Father of; C, control DNA.

The family of patient V.O.



The family of patient S.B.



The family of patient A.M.

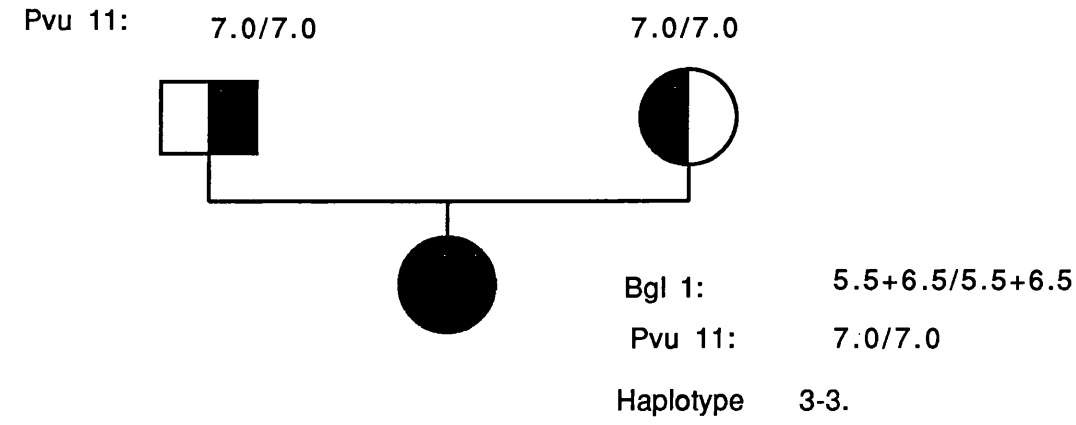


Fig 5.6. Haplotypes of fucosidosis patients.

5.4. Discussion.

The results have shown that none of the six fucosidosis patients in the study have either of the two previously described mutations in fucosidosis. Willems et al., (1991) found that these two mutations account for the disease in less than a third of fucosidosis families - in a survey of twenty one families, five families had the EcoR1 obliterated site mutation and one had the 3' deletion. The majority of fucosidosis patients therefore have a different mutation.

The seven patients identified by Willems et al. (1988b) with the nonsense mutation in exon 8 were all of haplotype 2-2. The mutation was not a de novo mutation in any of the families. This suggests that the mutation occured only once and was inherited from a common ancestor. It is probable that other patients with the same fucosidosis - causing mutation will also have the same haplotype. The two siblings with the 3' deletion were of haplotype 1-1. Willems et al.(in press) have found almost complete linkage disequilibrium between the 7.0kb Pvu11 and the 12.0kb Bgl1 allele (haplotype 1-1) and the 6.0kb Pvu11 and the 5.5+6.5kb Bgl1 allele (haplotype 2-2). The standard disequilibrium coefficient (r) was 1.0 for the patient population and 0.91 for Caucasian controls. Linkage disequilibrium between these RFLP's can be seen in the family V.O. (haplotype 2 - 2) and S.B. (haplotype 1 - 1). A.M., however, has haplotype 3-3. A.M. therefore has a different haplotype from any so far characterised in fucosidosis patients. This probably implies that A.M. has a different mutation from those represented by the haplotypes characterised previously, and that at least three more disease - causing mutations occur in fucosidosis.

In fucosidosis families bearing the EcoR1 mutation, amplification using primers 1 and 2 and subsequent digestion with EcoR1 would be a rapid, simple method for prenatal diagnosis, using small quantities of DNA. For patients where the mutation has not yet been characterised, the Pvu11 and Bgl1 polymorphisms could be used for prenatal diagnosis in families informative for linkage. The polymorphism information content (PIC) of the combined RFLP markers is 0.38 (Darby et al., 1988). Since both RFLP's are located in the FUCA1 gene, the frequency of homologous recombination will be very low. Prenatal

diagnosis on the basis of enzyme activity can be unreliable. Fucosidase activity in amniotic fluid fluctuates with gestation age (Wiederschain et al. 1971). Misdiagnosis of two affected twins as carriers on the basis of amniotic fluid enzyme assay has been reported (Matsuda et al., 1975). Carrier detection on the basis of enzyme assays is also unreliable because the activity range of controls overlaps that of heterozygotes. The low plasma activity polymorphism can complicate carrier detection based on enzyme activity if plasma or fibroblasts are used.

CHAPTER 6.

Detection of other mutations causing fucosidosis.

6.1.Introduction.

The six fucosidosis patients in this study were found not to possess either of the mutations previously characterised. These two mutations accounted for fucosidosis in only 6 out of 21 families (Willems et al., in press), suggesting that the majority of fucosidosis patients have a different mutation. Haplotype analysis suggests that at least 3 more disease-causing mutations are present in fucosidosis. In other lysosomal storage diseases several different disease-causing mutations have been found; at least 20, for example, in the β-hexosaminidase α-sub-unit gene causing Tay Sachs disease. Southern blotting has the potential of detecting gross gene alterations or point mutations that alter a restriction enzyme site over a large proportion of the gene. Mismatch analysis and direct sequencing of the amplified products of PCR have the potential of identifying any mutation but in a limited region of the gene. DNA from the 6 patients was analysed using these methods with the aim of detecting any new disease-causing mutations. At the beginning of the project sequence information was only available to construct primers to amplify the last exon.

6.2. Detection of mutations using Southern blotting.

No large insertions, deletions or gene rearrangements were detected on Southern blots of any of the six fucosidosis patients in the study. The fucosidosis phenotype therefore probably results from a small insertion, deletion or point mutation in these patients.

Point mutations and small insertions and deletions may fortuitously alter a restriction site thereby permitting detection by Southern blotting using the appropriate enzyme. Southern blot analysis of DNA from some of the patients was performed using the restriction enzymes,

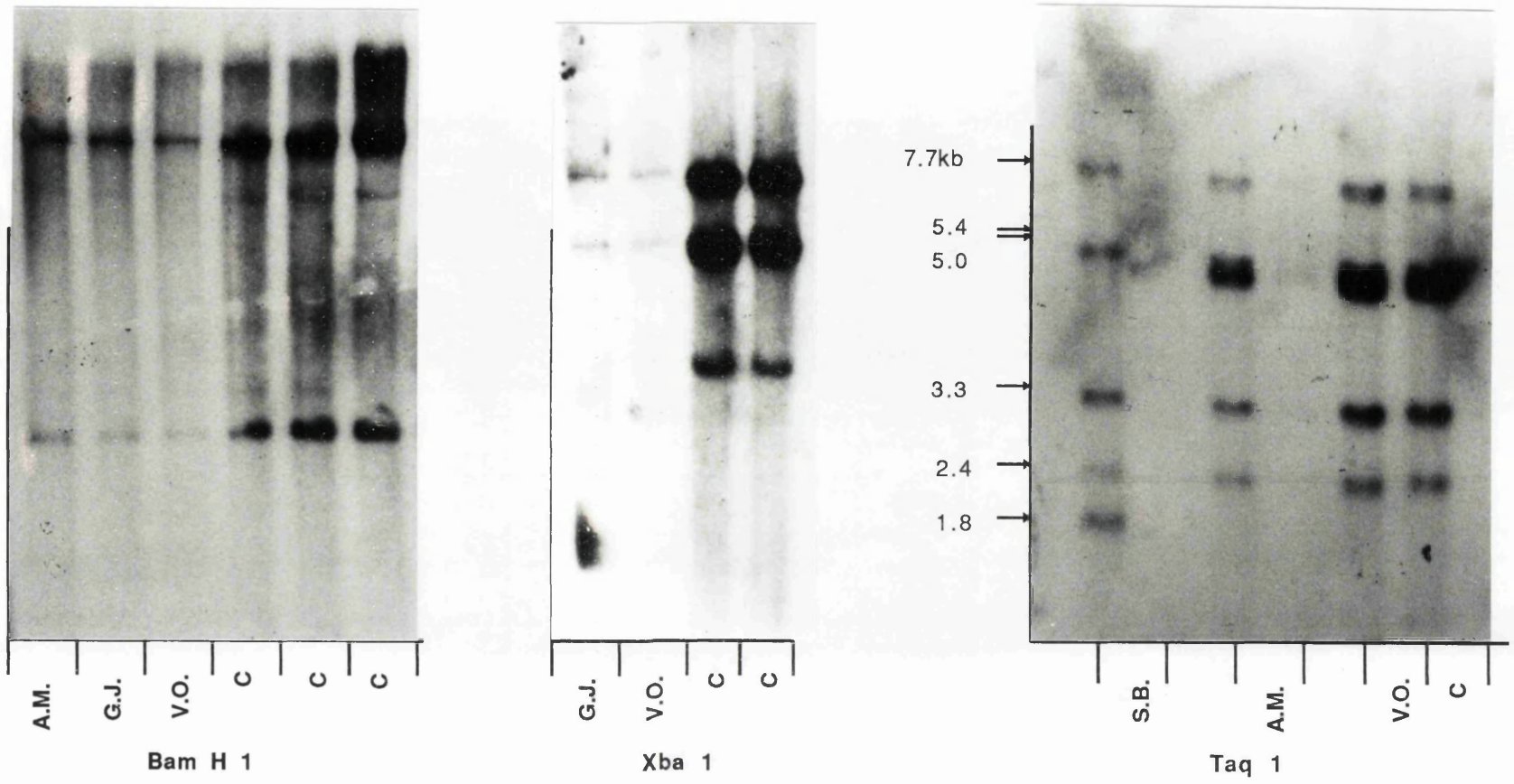


Fig 6.1 Southern blots of patient and control DNA hybridised to fucosidase cDNA probe A+B.

Bam HI and *Xba* I. The patients did not show an abnormal pattern of bands for either enzyme (fig 6.1). The same patterns of bands in controls and the patients had been seen with the restriction enzymes *Eco*R I, *Pvu* II, and *Bg*l I. (fig 5.2, 5.4 and 5.5).

Southern blots of DNA from V.O. and A.M. digested with *Taq* I had bands of the same size as controls - 7.7kb, 5.4kb, 5.0kb, 3.3kb and 2.4kb (fig 6.1). However an abnormal pattern was seen on a Southern blot of DNA digested with *Taq* I from patient S.B. The 5.0kb band was absent and a novel 1.8kb band was present (section 6.6).

6.3. Detection of mutations by restriction enzyme digestion of PCR products.

Some human genetic diseases result from small deletions or insertions that are too small to be detected by Southern blotting, for example many cases of cystic fibrosis (Kerem et al., 1989; Mathew et al., 1989). Amplification of a region containing a small insertion or deletion will produce amplification products differing in size by a few base pairs. If the amplification product is small enough, these size differences can be detected by differences in migration of the DNA fragments on polyacrylamide gels. If the product is too large, it can be cut with restriction enzymes prior to electrophoresis.

The amplified products of primers 1 and 3, covering exon 8 and the 3' non-coding region, from patients C.B., G.J., C.L., A.M., and V.O. and from controls were digested with *Bgl* II to generate three fragments of 447, 290, and 100 base pairs. The DNA was electrophoresed on polyacrylamide gels which were subsequently stained with ethidium bromide. As can be seen from fig 6.2 the three *Bgl* II-digested DNA fragments from each patient comigrated with similarly digested control DNA. Two extra bands of less than 100bp were also seen in the amplified product from patient C.B. These extra two bands were also present in DNA from control 1 and are not therefore associated with fucosidosis. The extra bands are probably an amplification artefact but could be a heterozygous polymorphism. Bands differing in size by 11bp or more were resolved on this gel as can be seen from the markers. These results suggests that no insertions or deletions of greater than 10bp occur in the last exon or flanking region in these patients. Small quantities of DNA were made available from fifteen more fucosidosis patients at this time (provided by Dr. P. Willems). The DNA was amplified with primers 1 and 2 and digested with *Pst* I and *Alu* I, to give fragments of 124, 75, and 58 base pairs. No size

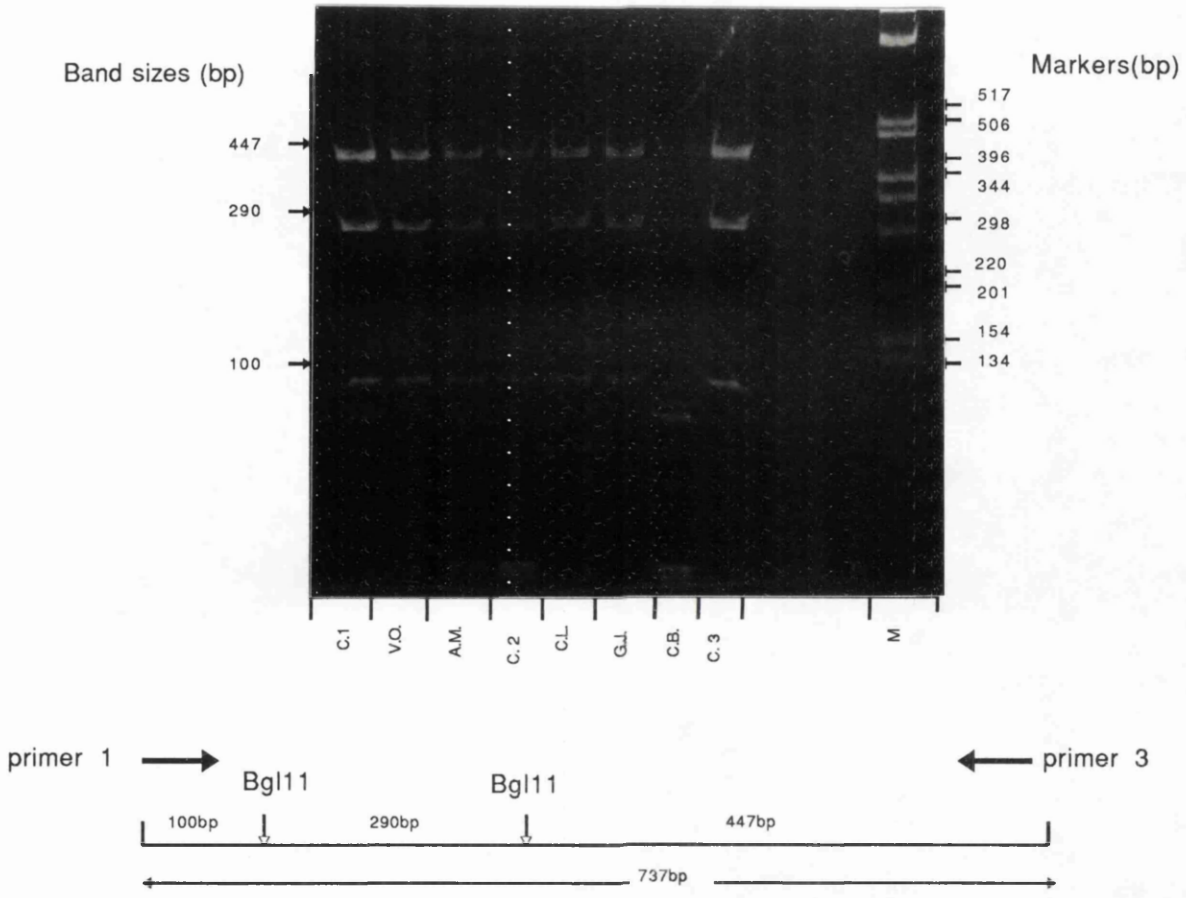


Fig 6.2. Polyacrylamide gel of the amplified products of primers 1 and 3 digested with *Bgl* II.

C.1, C.2, C.3 , fibroblast DNA from 3 different controls ; M, DNA size markers.

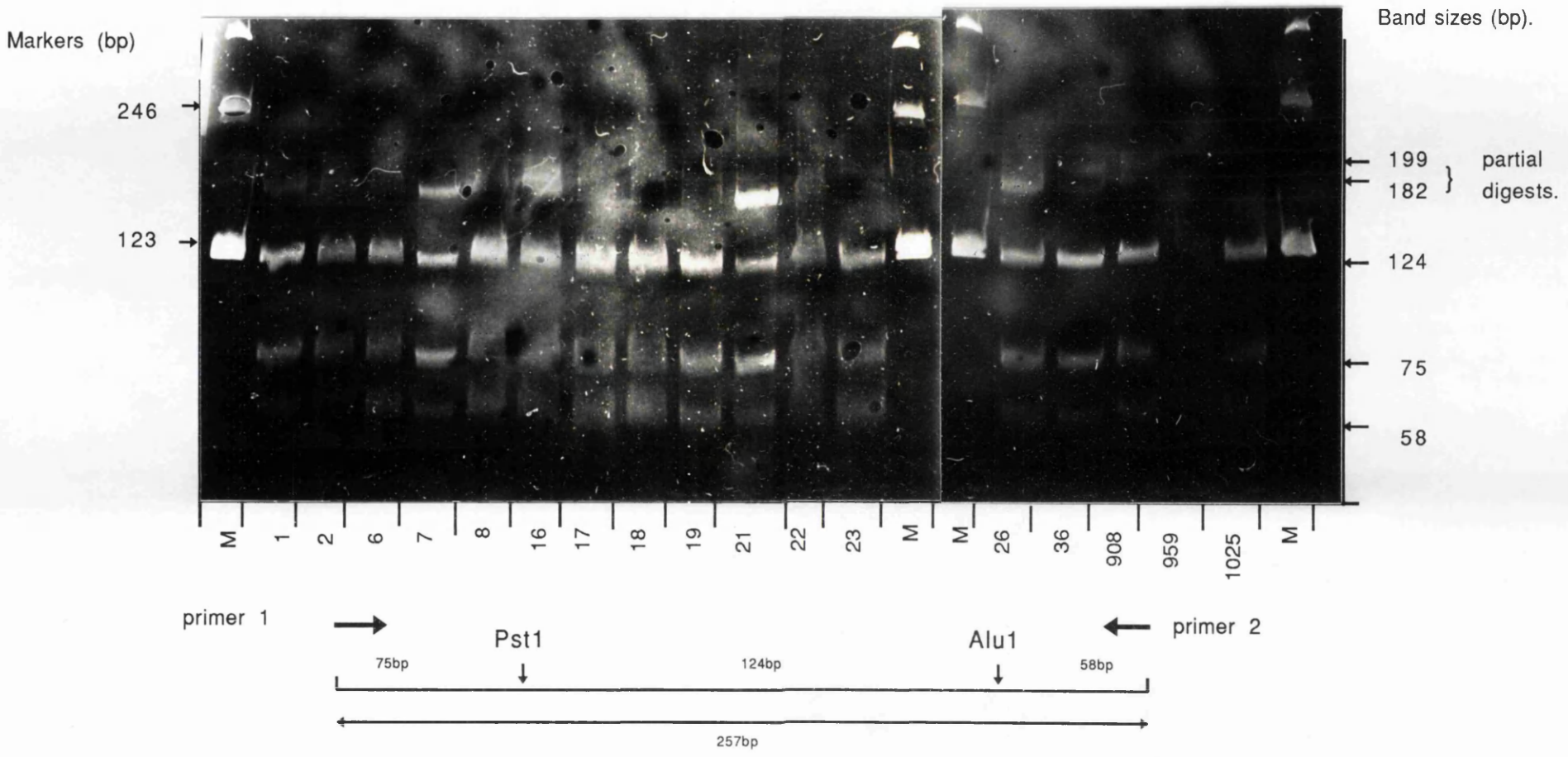


Fig 6.3. Polyacrylamide gel of the amplified products of primers 1 and 2 digested with Pst 1 and Alu 1.

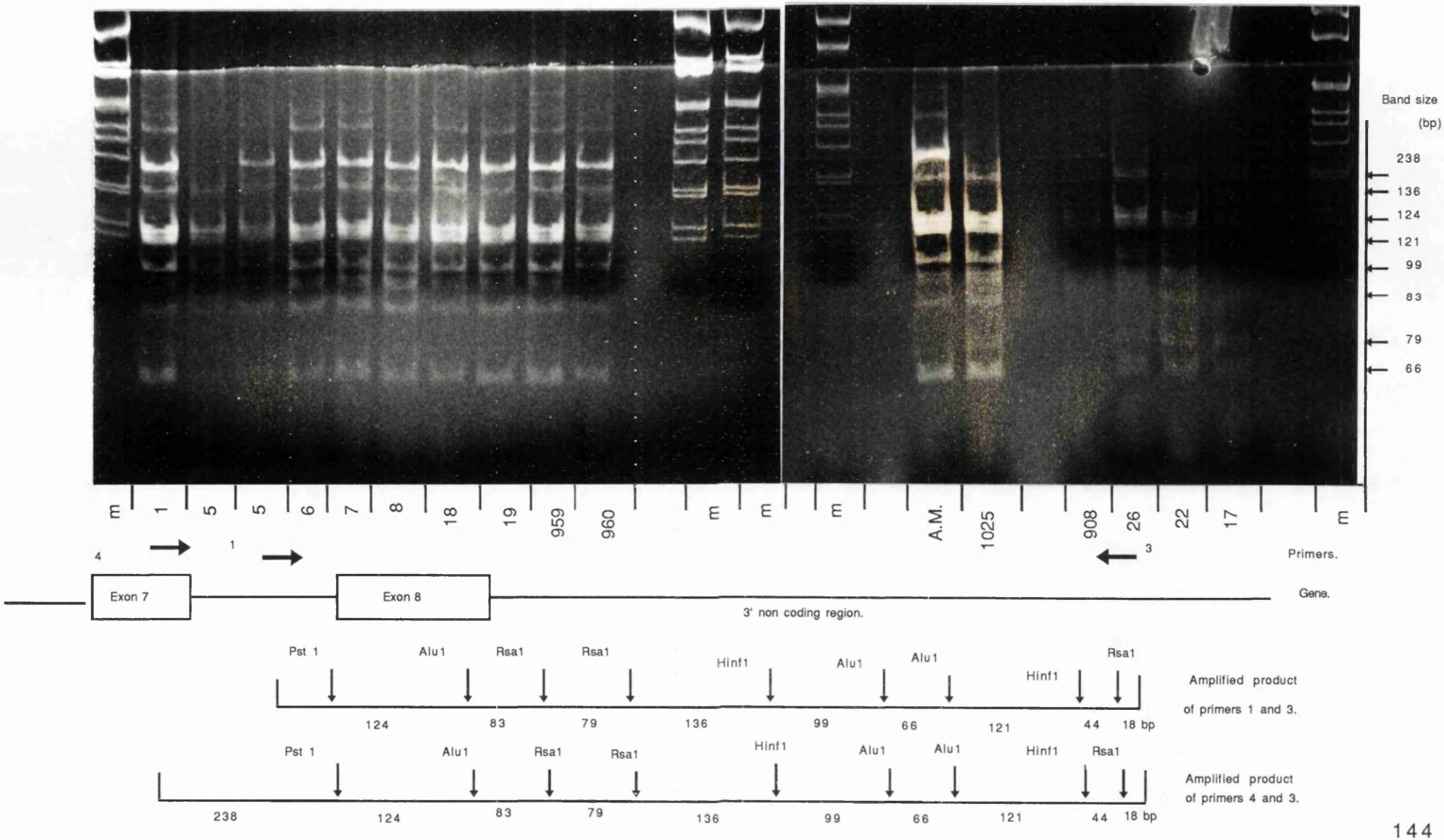


Fig 6.4. Amplified products of primers 1 and 3 and 4 and 3 digested with Alu1, Hinf1, Pst1 and Rsa1.

differences in the bands between the different patients were detected, following polyacrylamide gel electrophoresis (fig 6.3). However, very small size differences may not have been picked up as the bands were not well resolved on these gels.

Primer 4 (donated by Dr. P. Willems) was complementary to the 3' end of exon 7. Amplification with primers 1 and 3, and 4 and 3, gave rise to products of 784 and 946 base pairs respectively. The amplified products were digested with Alu1, Hinf1, and Rsa1. This generated fragments of 136, 124, 121, 99, 83, 79, 66, 44 and 18 base pairs with primers 1 and 3, and fragments of 238, 136, 124, 121, 99, 83, 79, 66, 44 and 18 base pairs with primers 4 and 3 (fig 6.4). The larger fragments probably result from partial digestion of the DNA by one or more of the enzymes. As can be seen from the figure, fragments differing in size by 3 base pairs could be resolved but no insertions or deletions were detected in any of the patients.

6.4. Amplification and mismatch detection.

6.4a). Basis of the technique.

Mismatch analysis has the potential to detect any point mutation, small insertion or deletion in the amplified product of a PCR reaction. Heteroduplexes are formed between radiolabelled control amplified product and the amplified product of the mutant DNA under investigation (fig 6.5). The heteroduplexes are then treated with the reagents osmium tetroxide or hydroxylamine, which modify single strands. At pH 6 hydroxylamine modifies the C5=C6 double bond in mismatched cytosine residues and labilises the ring to internal rearrangement and cleavage (Rubin and Schmid, 1980). Osmium tetroxide reacts specifically with mismatched thymidine residues resulting in the formation of 5,6 dihydroxythymidine, a change which leads to labilization of the polynucleotide chain at the position of the T residue (Friedman and Brown, 1978). The heteroduplexes are subsequently treated with piperidine to cleave DNA at all sites of modification. The mismatch reactions are analysed on a denaturing polyacrylamide gel which is then dried and autoradiographed.

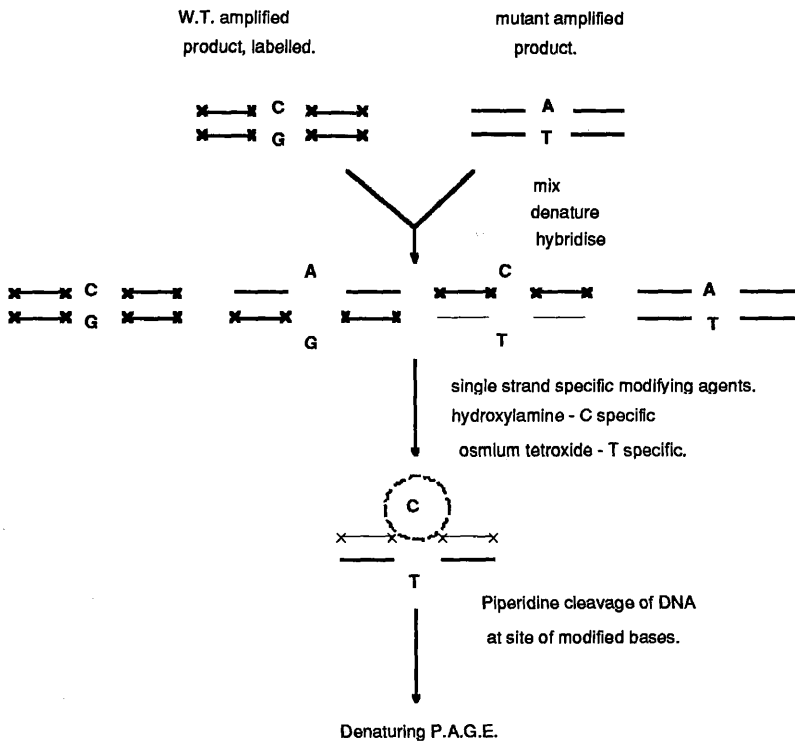


Fig 6.5. Diagram of the technique of mismatch analysis.

6.4.b). Labelling of wild type amplified product for use as a probe.

i). 5' end labelling of the probe.

The wild type amplified product was initially end-labelled with ^{32}P. It was then separated from unincorporated nucleotides using a Sephadex G-50 column, according to the method of Montandon et al., (1989). Incorporation of ^{32}P was found to be inadequate, however, when the probe was labelled in this way. A probe of high specific activity is required in this technique since a very small amount (10 ng) of labelled probe is used as unlabelled mutant DNA has to be in excess. During heteroduplex formation four different

combinations of amplification products are formed: probe DNA and mutant DNA will both self-anneal and probe and mutant strands combine in two different combinations.

W.T ------- ------- ****** ****** Mutant

------- ****** -------- ******

The labelled cleaved probed will consequently be approximately a quarter as intense as the uncleaved band for a homozygous mutation, and an eighth for a heterozygous mutation. Following mismatch analysis using end-labelled probe, a single band of low intensity representing uncleaved probe was seen. Any bands of cleaved probe would not have been detected since the intensity would have been too low.

ii). Incorporation of ^{32}P during PCR to label the probe.

The wild type amplified product was consequently labelled by incorporation of ^{32}P during the PCR. As well as greatly increasing the amount of ^{32}P incorporated into the product, this technique has the advantage of giving two bands of cleaved product on the autoradiogram rather than the single band seen when ^{32}P end labelling is used. The point of cleavage, and consequently the mutation, can therefore be localised more accurately. The probe, labelled in this way, was initially separated on a Sephadex G-50 column. However, after running this probe on a polyacrylamide gel, and autoradiography, many smaller bands of low intensity in addition to the main band were seen, probably resulting from non specific priming of PCR. The labelled probe was therefore excised from an agarose mini gel and the DNA eluted from the agarose using "Geneclean". This gave rise to a single band of high intensity on autoradiograms of polyacrylamide gels.

Probe DNA labelled by incorporation of ^{32}P during PCR and purified by agarose gel

electrophoresis was therefore used in all subsequent analyses.

6.4.c). Preparation of unlabelled amplified products.

DNA from C.B., G.J., C.L., A.M., and V.O. and a control was amplified using primers 1 and 2, or, 1 and 3, and products were analysed on an agarose minigel. The DNA was purified from the gel using "Geneclean". This DNA was hybridized to the labelled probe, and used in the mismatch reaction. Labelled wild type amplified product hybridized to unlabelled wild type amplified product was used as a negative control in all the analyses. DNA from a fucosidosis patient heterozygous for the obliterated EcoR1 site mutation in exon eight was used as a positive control.

6.4.d). Results.

As can be seen from fig 6.6 only a single band representing labelled uncleaved probe is present in all the patients and the negative control, using primers 1 and 2 and after modification with hydroxylamine. No bands representing cleaved wild type amplified product and mismatched heteroduplexes were seen. When the amplified product of a heterozygote for the obliterated EcoR1 site mutation was used, two additional bands, of approximately 166 and 91 base pairs, could be seen following hydroxylamine modification. These correspond to cleaved probe DNA, cleavage occurring at the point of mismatch in the heteroduplex. No mismatches were detected in patients C.B., G.J., C.L., A.M., or V.O. in amplified products of primers 1 and 3, with hydroxylamine or osmium tetroxide modification. This suggests that no point mutations, insertions or deletions are present in the amplified region between primer sequences 1 and 3, in these patients. However as a positive control for modification with osmium tetroxide was not used it is technically possible that thymidine mismatches may not have been detected.

A novel band was seen on a sequencing gel of exon eight of A.M., representing a transition of A-G. To verify this mutation the amplified product of DNA from A.M. was labelled by incorporation of ^{32}P during PCR, and hybridized to unlabelled control DNA. Labelled DNA from patient A.M. hybridized to unlabelled DNA from A.M. was used as a

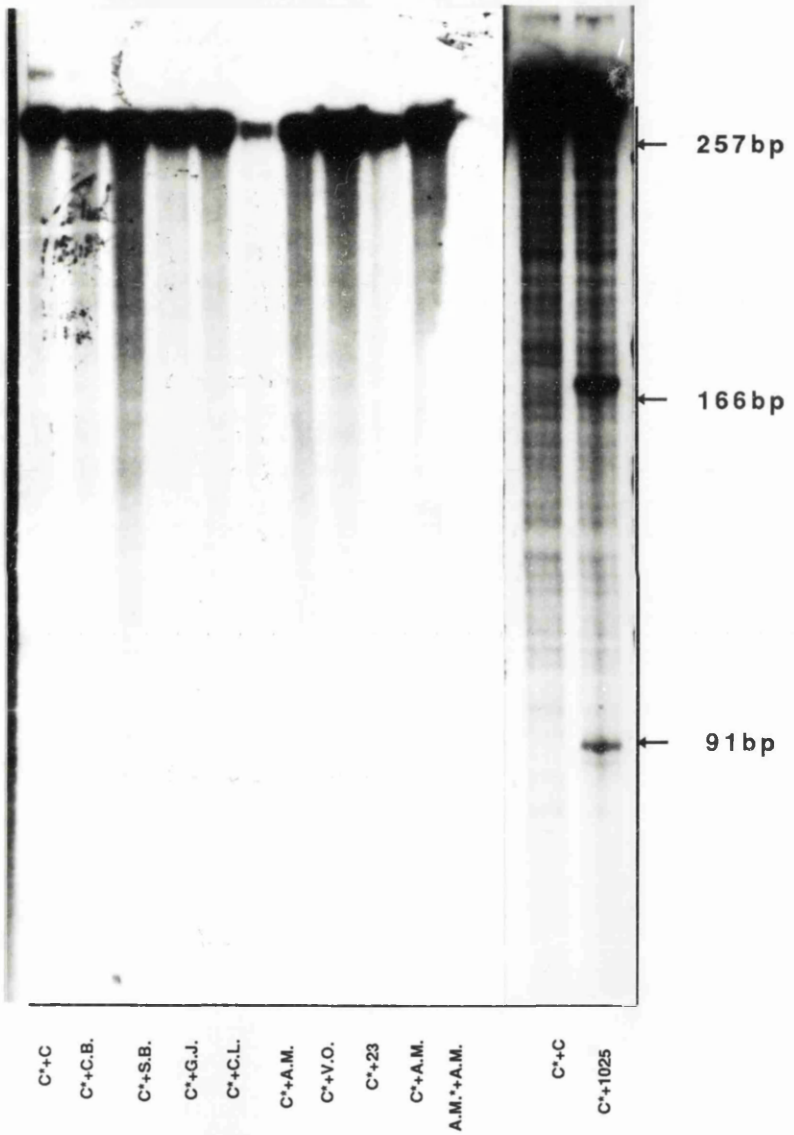


Fig 6.6. Mismatch analysis of exon 8.

C, control; C*, labelled control; 1025; fucosidosis patient heterozygous for the EcoR1 obliterated site mutation.

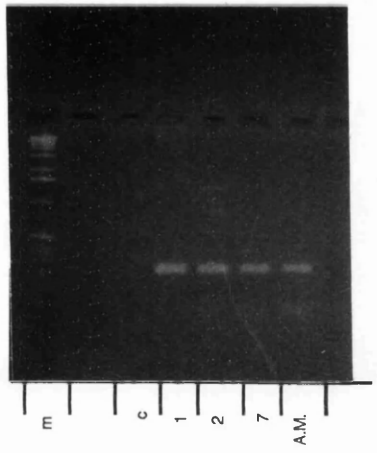
negative control. No cleaved bands were seen after hydroxylamine modification. This implied that the new band seen on the sequencing gel was an artefact. When this region of A.M. DNA was sequenced a second time, the new band was not present confirming that the new band was an artefact.

6.5. Direct sequencing of amplified products.

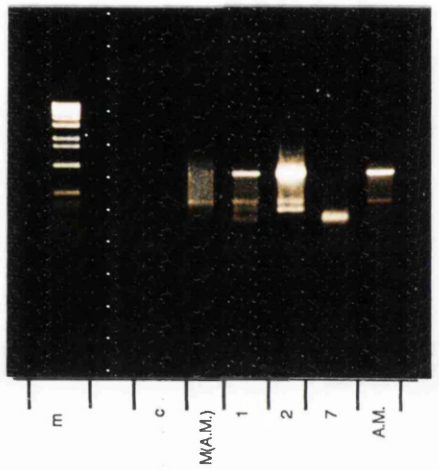
6.5.a). Development of the technique.

At the same time as the mismatch technique was being developed, the amplified products of primers 1 and 2 were sequenced directly. Genomic DNA from the six patients was amplified with primers 1 and 2. 15% of the product was analysed on an agarose minigel. If a single sharp intense band was seen, implying a specific amplification, the remaining DNA product was extracted with phenol and chloroform and precipitated with ethanol. The pellet was used in a second amplification with primer 2 only, to generate a single stranded product. 15% of this amplification was analysed on an agarose minigel. If two or more sharp bands were seen on the minigel (fig 6.7), the remaining product was phenol and chloroform extracted and precipitated. Half of this pellet was used in a sequencing reaction using primer 1.

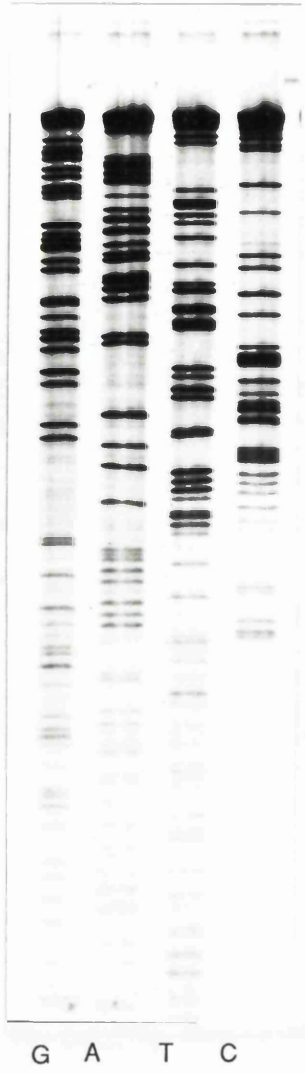
In some cases, genomic DNA was not available, only amplified products of primers 1 and 2, or 1 and 3 cut out of an agarose minigel. In these cases the pieces of gel containing the amplified product was frozen and thawed in TE twice. 15% was reamplified in a PCR with primers 1 and 2 for 11 cycles. The double stranded PCR product was then used as for genomic DNA sequencing. Amplification using primers 1 and 2 from a PCR product of primers 1 and 3 increased the specificity.



a). Amplified products of primers 1 and 2.



b). Amplified products from a) reamplified with primer 2 only.



c). Sequence of A.M.

Fig 6.7. Diagram to show the preparation of template for sequencing.

c, control PCR with no DNA; 1,2,7, A.M., DNA from fucosidosis patients.
M(A.M.), DNA from the mother of patient A.M. m, DNA size markers (1kb ladder).

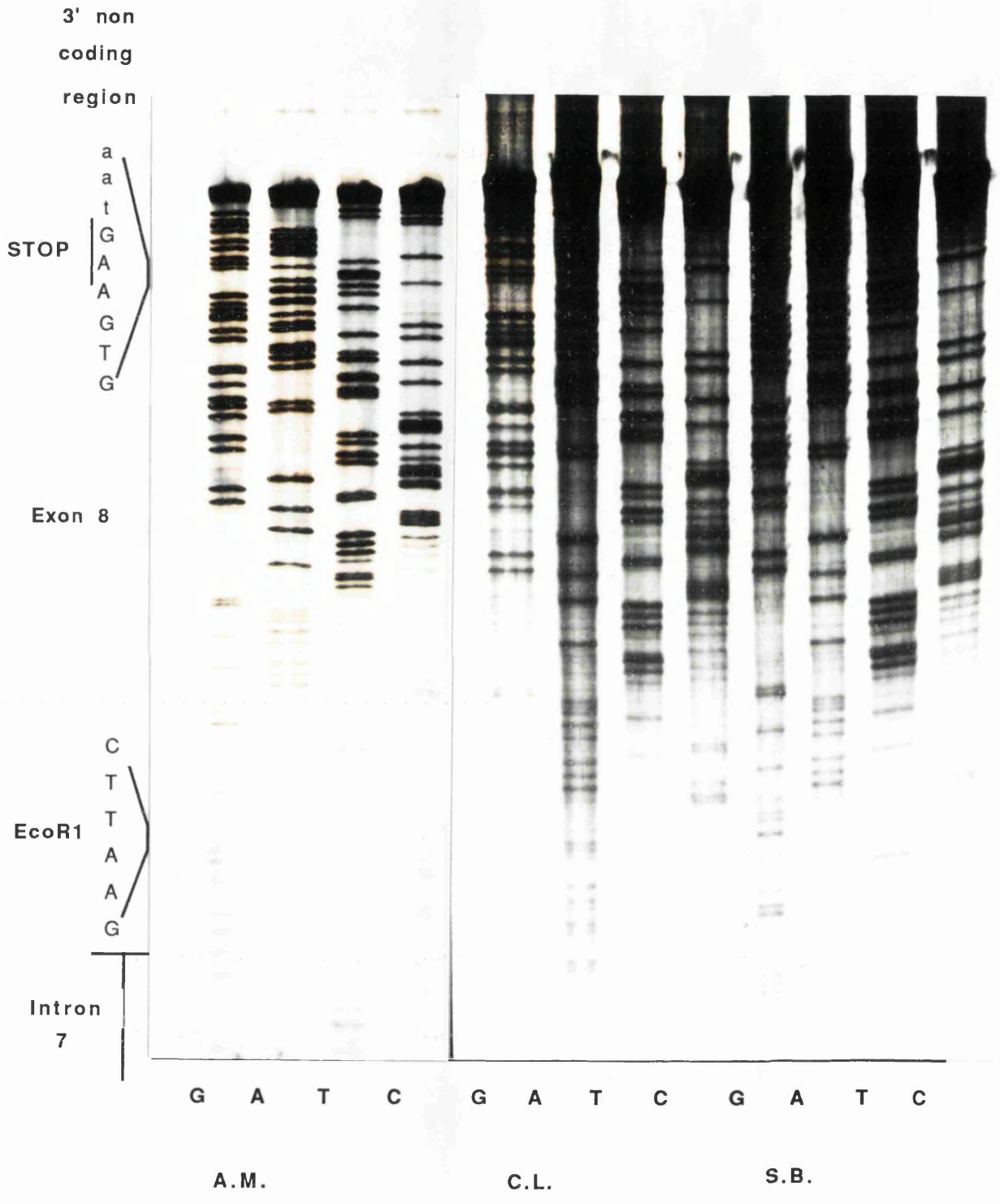


Fig 6.8. Sequence of exon 8 of the fucosidase gene from 3 fucosidosis patients.

6.5.b). Results.

The amplified products of C.B., S.B., G.J., C.L., A.M., and V.O. were sequenced with primer 1 . No change in sequence from that of controls was seen (fig 6.8). DNA from 5 other fucosidosis patients was also sequenced; this also was identical to the control sequence (not shown).

6.6. Characterization of the Taq 1 polymorphism in the DNA of patient S.B.

An abnormal pattern of bands was seen on a Southern blot of DNA from patient S.B. digested with Taq 1. The normal 5kb Taq 1 fragment was absent and a novel 1.8kb band was present (fig 6.1). The new pattern seen on the Taq1 Southern of S.B. could be a result of a mutation in the fucosidase gene of S.B. which generates a new Taq1 site within the 5.0kb Taq1 fragment. S.B. must be homozygous for this mutation since the 5.0kb fragment could not be detected at all, eliminating the possibility of heterozygosity.

6.6a). Cosegregation of the Taq 1 polymorphism with fucosidosis in the family of S.B.

To determine whether the mutation cosegregates with fucosidosis in this family, a Southern blot of Taq1-digested DNA from the parents and siblings was made.

As can be seen from fig 6.9 the parents of S.B., who are both obligate heterozygotes for fucosidosis, were also heterozygous for the mutation with both the 1.8kb and the 5.0kb Taq1 bands. Three of the siblings (A.B., H.B. and N.B.) were also heterozygous for the mutation, whereas two siblings (D.B. and Sa.B.) lacked the mutation in either allele. The presence of the mutation correlates with fucosidase enzyme activities (fig 6.10).

The mutation giving rise to the new Taq1 site must be 1.8kb from the 5' or 3' end of the 5.0kb Taq1 fragment. Another fragment of 3.2kb must also be generated - this is not seen on the Southern. This may be because the 3.2kb fragment contains only intronic sequences (which would not hybridize to the cDNA probe) or the 3.2kb fragment comigrates with the 3.3kb band present in controls.

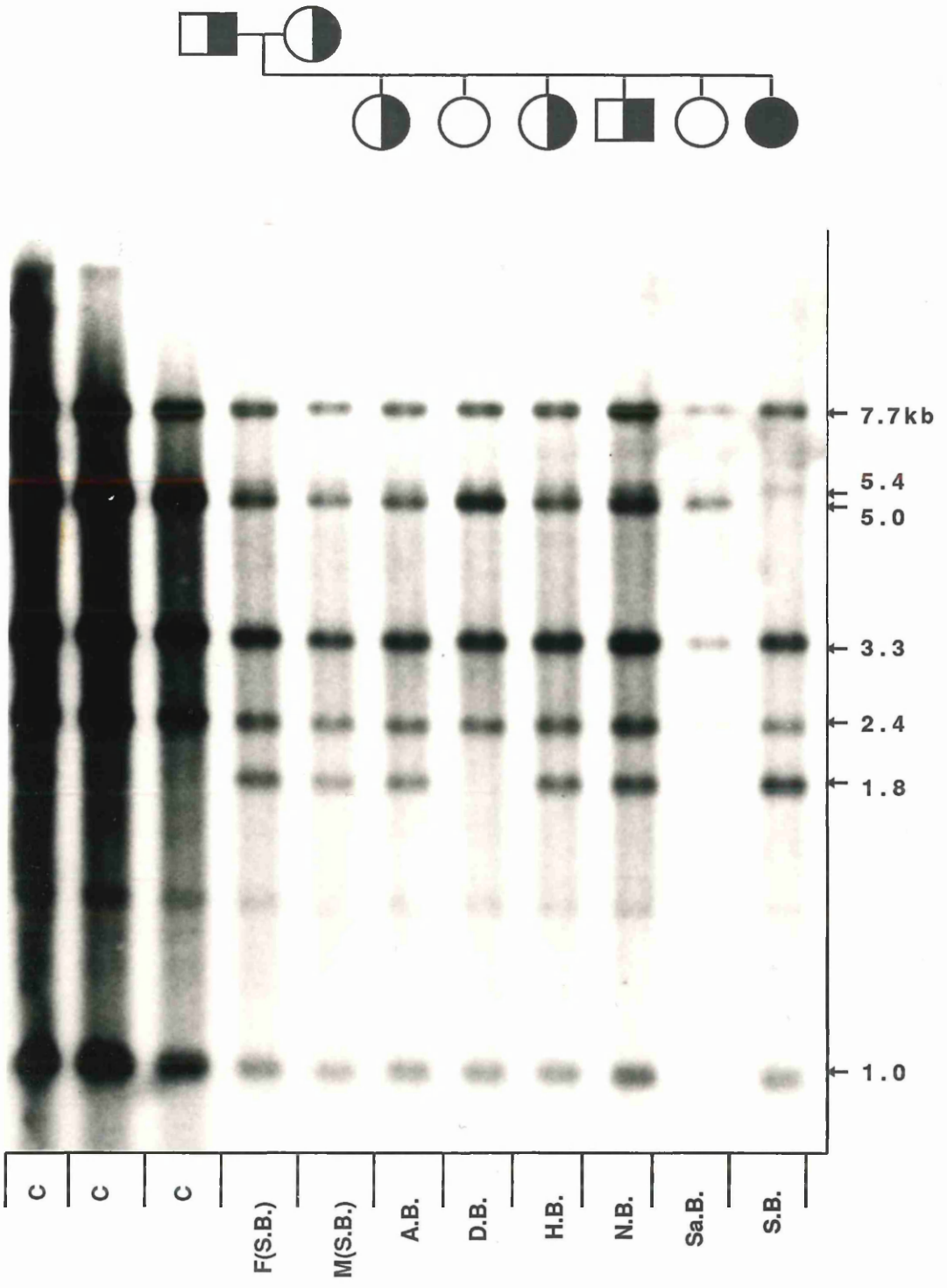


Fig 6.9. Southern blot of DNA from the family of S.B. digested with Taq1 and hybridised to fucosidase cDNA probe B.

F(S.B.), father of S.B.; M(S.B.), mother of S.B.;

A.B., D.B., H.B., N.B., Sa.B., siblings of S.B.

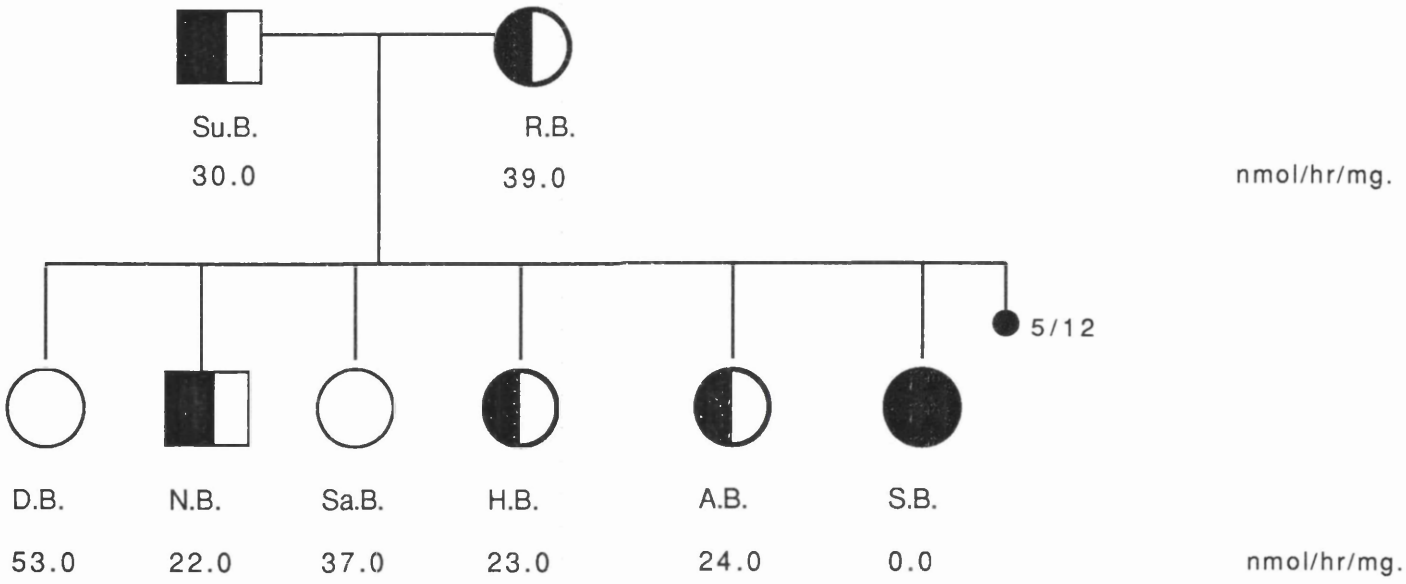


Fig 6.10. Correlation between the novel Taq 1 site and leukocyte activity in the family of S.B.

Taq 1 site present.

6.6b). Experiments to localise the new Taq 1 site.

The new Taq 1 site appears to cleave the 5kb Taq 1 fragment into a 1.8kb and a 3.2kb fragment. The new Taq 1 site is therefore located within the region of the fucosidase gene represented by the 5kb fragment. Attempts were therefore made to localise the 5kb Taq 1 fragment within the gene.

Bands of 7.7kb, 5.4kb, 5.0kb, 3.3kb, 2.4kb and 1.0kb are seen on Southern blots of DNA from controls digested with Taq 1 and hybridized to fucosidase cDNA probe B. The 7.7kb band represents the fucosidase pseudogene on chromosome 2 (Willems et al., in press).

Probe C hybridizes only to the 2.4kb fragment (fig 6.11). Probe C corresponds to the 3'end of exon 8 and the 3'non-coding region. No Taq 1 sites are present in exon 7, intron 7, or exon 8 according to the published sequence. The 2.4kb Taq 1 fragment therefore spans exons 7 and 8. The 2.4kb fragment is also absent from patients with the 3' deletion (Willems et al. in press) supporting this interpretation of the results.

The 5.4kb Taq 1 band is much less intense in comparison with the other bands when hybridized to probe B, whereas the Taq 1 bands including the 5.4kb bands are of equal intensity on Southern blots hybridized to probe A + B (fig 6.11). This suggests that the 5.4kb fragment spans exon 3. The Hinc 2 site separating probe A and B is in exon 3, 5bp upstream from the 3' end of exon 3. Probe B will therefore only have 5bp in common with exon 3 and would hybridize to it inefficiently. Probe A + B spans the whole of exon 3.

A Taq 1 site is present 158bp upstream of exon 4 in control DNA, according to the published sequence. Exon 4 is 106bp long. The new site cannot therefore be in exon 4 since the new Taq 1 site must be either 1.8kb or 3.2kb from a Taq 1 site present in DNA from controls. The 5kb Taq 1 fragment must therefore span either exon 5 or 6. Following this reasoning exons 5 and 6 were chosen as a starting point to determine the exact position of the new Taq 1 site.

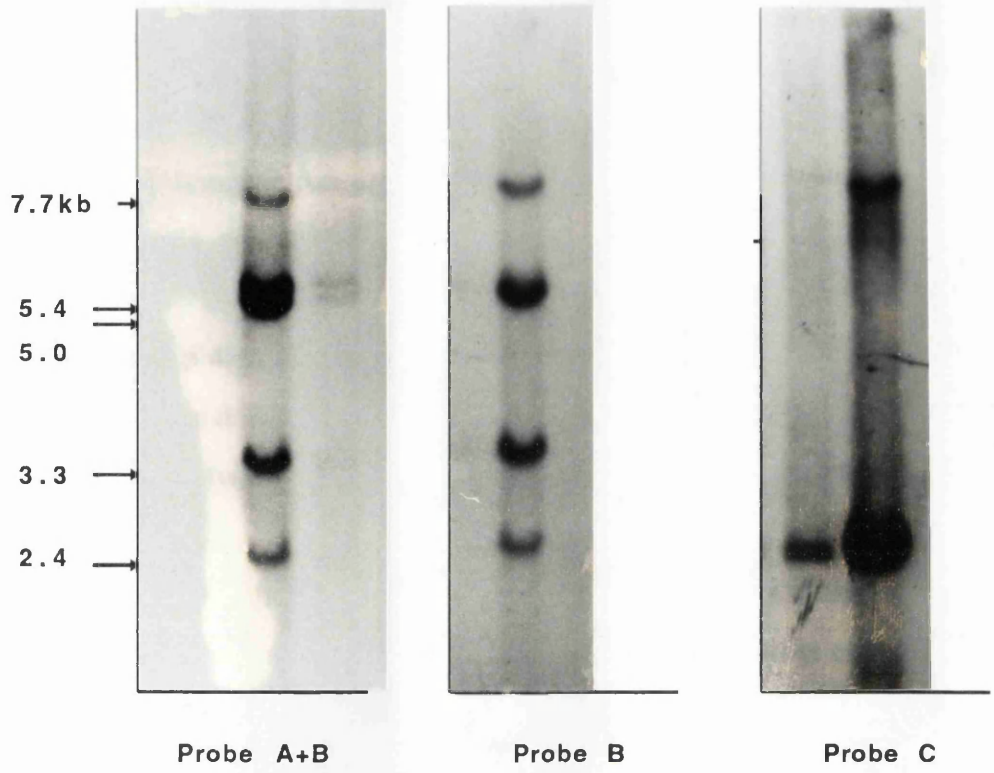
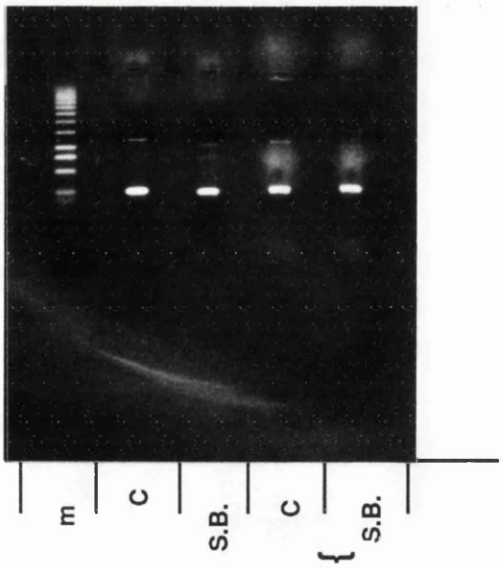


Fig 6.11. Southern blots of DNA from controls digested with Taq1 and hybridised to adjacent fucosidase cDNA probes.



Digested with Taq1.

Fig 6.12.Amplified products of primers 9 and 10 flanking exon 6.

i). Amplification of exon 6.

DNA from patient S.B. and controls was amplified by PCR using primers complementary to the intron sequences flanking exon 6 (primers 9 and 10) and the amplified products were subjected to Taq 1 digestion. The amplified products of DNA from both S.B. and controls failed to cut with Taq 1, showing that the new Taq 1 site is not in exon 6, (fig 6.12).

ii). Amplification of exon 5.

DNA from controls and patient S.B. was amplified using primers specific for the 5'and 3'ends of exon 5 (primers 7 and 8). This failed to cut with Taq 1 showing that the new Taq 1 site is not present in exon 5 (fig 6.13a).

When the relevant sequence information became avaliable a primer was constructed which was complementary to a region within intron 5 (primer 12). This was used, together with a primer specific for the middle of exon 5 (primer 11), to amplify across the 5' splice site of intron 5. DNA from controls and patient S.B. was amplified using these primers to produce a 167bp product. The amplified product of DNA from controls failed to cut with Taq 1 whereas the amplified product from S.B. was cut into approximately 100bp and 67 bp fragments (fig 6.13b). The amplified product of exon 5 failed to cut with Taq 1 whereas the overlapping product spanning the 3' end of exon 5 and the first 67 bases of intron 5 did cut with Taq 1. The new site must therefore be within the first 41bp of intron 5 (the 3' primer was 26bp long).

Taq 1 cut the 167bp product into fragments of approximately 100bp and 67bp. The new site must therefore be approximately 100bp 3' from the 5' primer. The 5' end of intron 5 begins 100bp from primer 11 and has the following sequence:

TCGgt

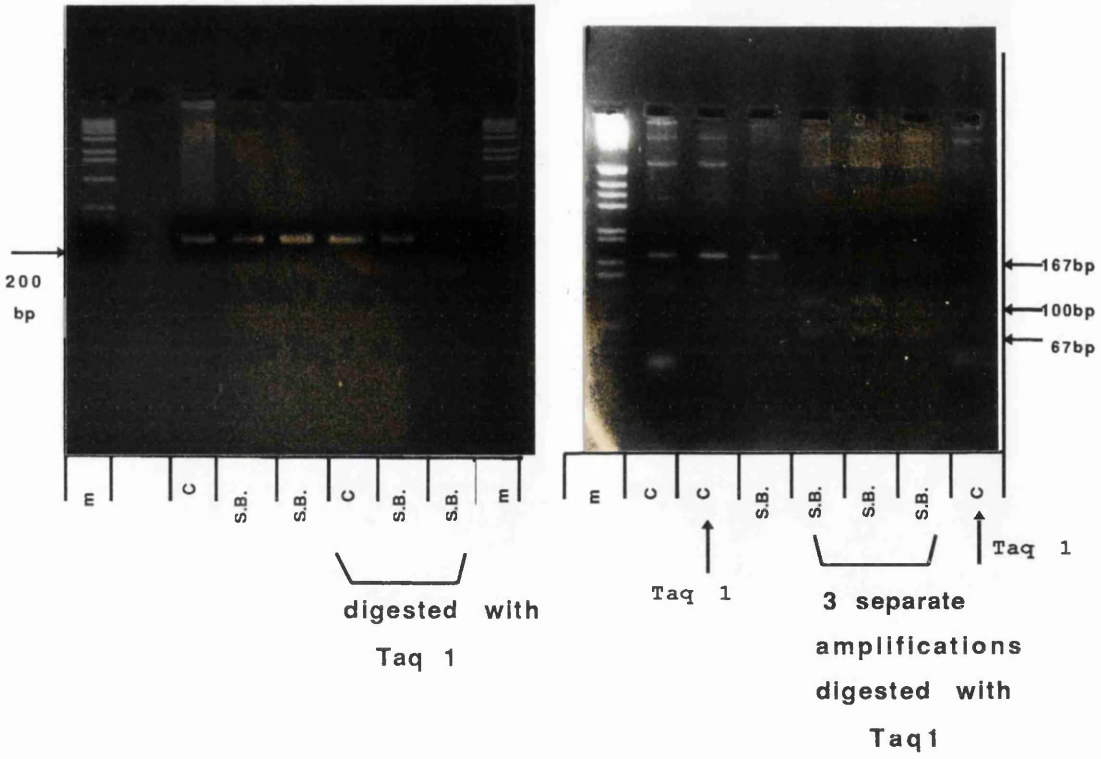


Fig.6.13a.
Amplified products of primers 7 and 8 digested with Taq 1.

Fig.6.13b
Amplified products of primers 11 and 12 digested with Taq 1.

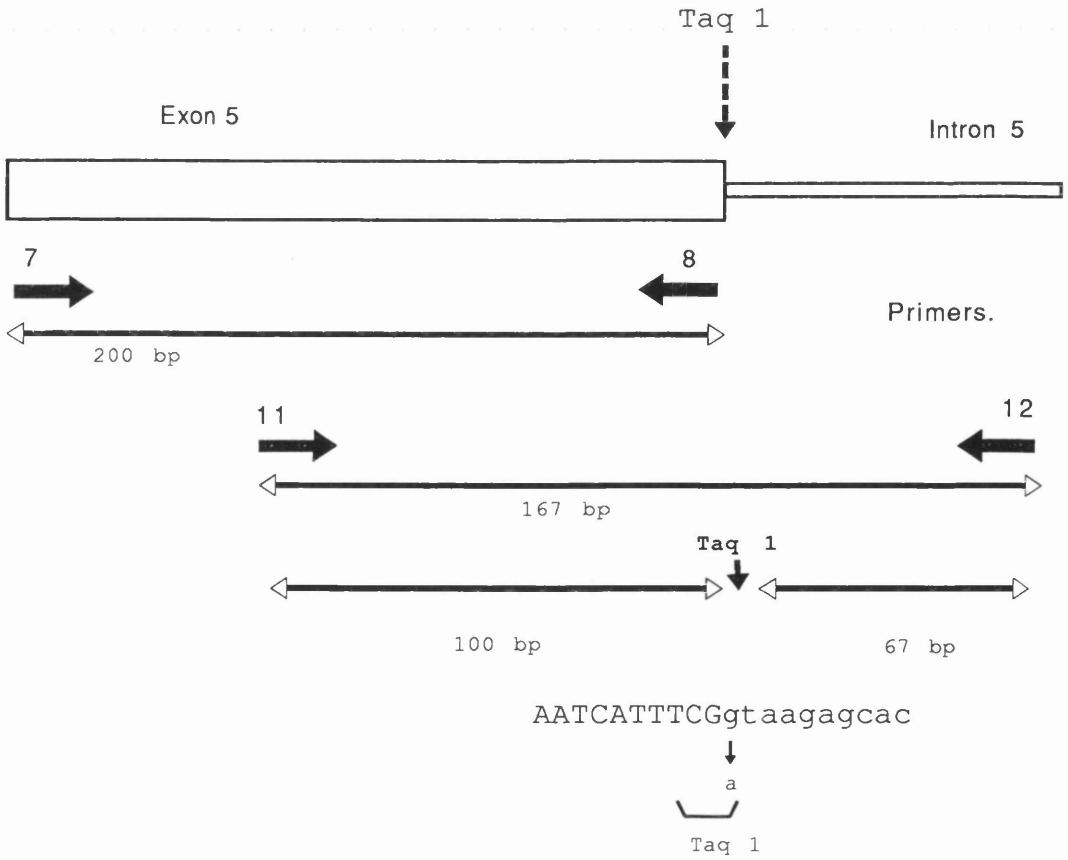


Fig 6.13c. Diagram to show the position of the mutation in patient S.B.

A single base substitution of G to A at the first position of intron 5 would generate a Taq 1 (TCGA) site at this position (fig 6.13c).

The exon 5 / intron 5 boundary region of DNA from patient S.B. and a control was sequenced and a G -> A transition at the first residue of intron 5 was found in DNA from patient S.B. (fig 6.14).

6.7. Discussion.

Mismatch analysis has the potential to detect all mutations. However only a relatively small region, limited by the length of sequence easily amplified, and defined by the primers, can be analysed at one time. Mismatch analysis can be used to localise a mutation approximately.

Sequencing identifies the exact location and base change in the mutation. Direct sequencing eliminates the requirement of cloning, and is therefore less time consuming. Also the high error rate of Taq DNA polymerase (1 misincorporation per 10,000 bases) is less likely to be a problem with direct sequencing than sequencing following a cloning step.

Mismatch analysis and sequencing showed that no mutations were present in exon 8 or flanking sequences from any of the six patients in the study or in the DNA from five other fucosidosis patients.

A polymorphism giving rise to a new Taq1 site was found in the family of S.B. This polymorphism is either in strong linkage disequilibrium with the mutation causing fucosidosis or is the disease-causing mutation itself since it is inherited concordantly with the disease. This Taq1 polymorphism has not been found in 12 controls (4 from this study and 8 from the study of Darby et al., 1988) supporting evidence that this is a disease-causing mutation. The strongest evidence for the polymorphism being the mutation causing fucosidosis in this family is the nature of the mutation itself. The consensus sequence for 5'splice sites is as follows:

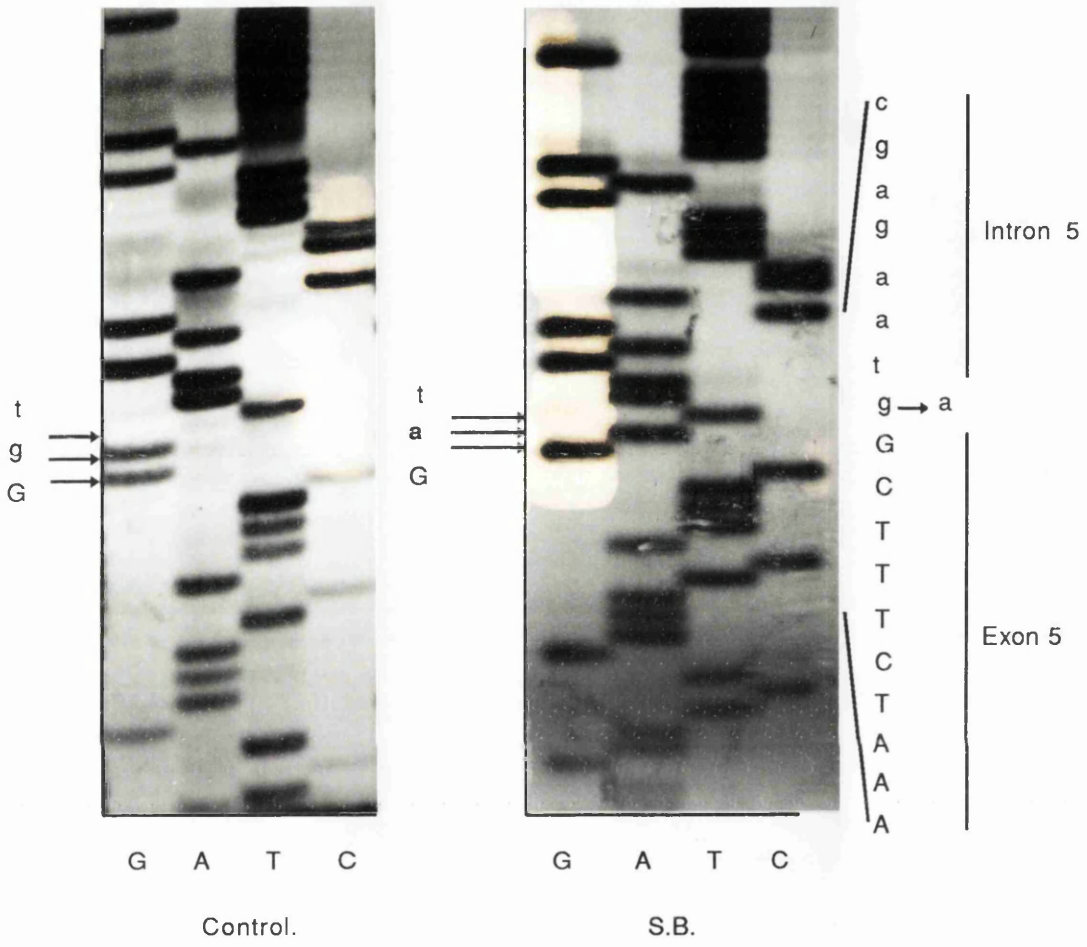


Fig.6.14. Sequencing of the splice site mutation at the exon 5/ intron 5 boundary in DNA from patient S.B.

in which the G and U at the first and 2nd positions of the intron are the most highly conserved bases (Jacob and Gallinaro 1989). Only 3 out of a total 1893 5' splice sites analysed by Shapiro and Senopathy (1987) did not have a guanine at position +1 of the intron. These 3 introns were all from immunoglobin genes. The 5'consensus sequence shows complementarity to the 5' terminal region of the small nuclear RNA U1 which is thought to bind to it. Splicing occurs by a 2 step mechanism. Cleavage initially occurs at the 5' donor site and the 5' end of the intron is joined to the 2'OH of an adenosine within the branch site region of the intron giving rise to a lariat form of the intron. The second reaction results in ligation of the exons and release of the lariat intron. In vitro experiments involving mutations in the splicing regions of the large beta globin intron have shown that a GT-> AT mutation at position +1 of the intron does not prevent cleavage of the 5' splice site region but does prevent 3' cleavage and exon joining (Aebi et al., 1986). The G -> A transition therefore prevents the second step of splicing and results in accumulation of a lariat intermediate in vitro.

Disruption of the 5' donor splice site of intron 5 of patient S.B. would probably result in the use of one or more alternative splicing mechanisms. Which alternative mechanism is used in this case is probably determined by the order of splicing of the introns in the fucosidase gene and the sequence of the rest of intron 5. The same G -> A mutation as that found in patient S.B. has been found in 3 different introns of the gene for type III procollagen in 3 different Ehlers Danlos patients (Kuivaniemi et al., 1990). The 3 identical mutations induced very different effects on splicing. The G+1 -> A mutation in intron 16 caused skipping of the preceding exon in 71% of the transcripts. The same mutation in intron 42 resulted in complete use of a cryptic splice site and consequent insertion of 30 nucleotides in frame into all the mature mRNA. An identical mutation in intron 20 resulted in the use of a cryptic splice site (53% of transcripts) and retention of all of the intron sequences in the mature mRNA (34%). The same splice site mutation therefore results in exon skipping in some cases only. This probably reflects the different relative rates at which adjacent introns are normally spliced. If, for example, the U2 and U5 snRNP complex bound to the 3' end of

intron 5 in the fucosidase gene before a similar complex bound to the 3' end of the upstream intron, the subsequent processing would lead to skipping of exon 5. This would result in deletion of 201 bases from the mRNA. The transcript downstream of that coded by exon 5 would remain in frame. If the U2 and U5 complex bound to the 3' end of intron 4 before intron 5 then exon skipping would not occur. Cryptic 5' splice sites could be used in intron 5 if sequences close to the consensus sequence were present, resulting in insertion of part of intron 5 into the mature transcript. Alternatively the whole of intron 5 may be inserted into the mRNA. Insertion of intronic sequences may result in a frameshift and premature termination at a stop signal in that frame. The same 5' splice site mutation resulted in exon skipping producing a truncated protein product in phenylketonuria, low amounts of truncated mRNA in Tay Sachs, and no detectable mRNA in metachromatic leukodystrophy (Dilella et al., 1986; Alki et al., 1991; Polten et al., 1991).

The mutation generating a new Taq1 site in patient S.B. has not been found in 22 other fucosidosis patients (2 from this study and 20 from that of Willems et al., in press), so it is either only present in this family or is rare.

The patient S.B. is homozygous for the mutation. This is to be expected since consanguinuity has been reported in this family. Sa.B., a sibling of patient S.B., lacked the splice site mutation in either allele and is therefore homozygous normal. However, she would probably be designated a carrier of fucosidosis on the basis of leukocyte fucosidase activity (Chapter 3). This illustrates the unreliability of carrier detection on the basis of enzyme activity.

CHAPTER 7

Canine fucosidosis.

7.1.Introduction.

Canine fucosidosis is a degenerative neurovisceral lysosomal storage disease found in English Springer spaniels. The disease results from a deficiency of α-L-fucosidase and is characterised by storage of fucoglycoconjugates (Abraham et al., 1984). Programmes have been established in England and Australia with the aim of eradicating the disease from the breeding stock (Barker et al., 1988b). These eradication programmes are based on heterozygote detection using enzyme assays. Enzymatic methods are unreliable for carrier detection because the ranges of activities for controls and heterozygotes overlap. DNA-based tests are not as yet available since very little is known of the canine fucosidase gene.

Characterization of the deficiency in dogs is also of special importance since it can be used as an animal model for human fucosidosis. Allogeneic bone marrow transplantation (BMT) offers a means of permanent enzyme replacement for lysosomal storage disease. However, BMT entails significant risks to the patient of graft versus host disease and graft rejection with resultant aplasia. Its use in patients with neurological disorders is controversial due to the difficulty of delivering lysosomal enzymes across the blood brain barrier. It is therefore essential to establish the effect of such therapies on animal models of the disease before treatment of affected children. Fucosidosis dogs has been used as a model of the human disease to determine the efficacy of enzyme replacement therapy (Taylor et al., 1988, 1989). They will also be used in trials of gene replacement therapy (Anson et al., 1990). The biochemical and genetic basis of canine fucosidosis was investigated in nine English Springer spaniels.

7.2. α–L-fucosidase enzyme activity.

The fucosidase activity in leukocytes and plasma of English Springer spaniels was

Leukocyte and plasma alpha-L-fucosidase activity.

			Plasma nmol/hr/ml.	% of control.	Leukocyte ratio. fuc/hex	% of controls.
Control.						
(n=192)			2.24∓0.48	100%	5.86∓1.53	100%
Obligate heterozygotes(n=6)			1.12∓0.21	50%	3.09∓0.71	
Dog.						
	A	m	1.1	49%	3	51.20%
	E	f	2.3	103%	5.2	88.70%
	G	m	1.1	49%	2.9	49.50%
	J	f	0	0%	0	0%
	L	f	1.8	80.30%	6.2	105.80%
	P	m	3.8	169.60%	3	90.40%
	S	m	2.4	107%	4.7	80.20%
	T	m	1	44.60%	ND	
	W	f	0	0%	0	0%

f=female m=male Fuc= alpha-L-fucosidase. Hex= beta-hexosaminidase.

Table 7.1

assayed using the synthetic substrate 4-methylumbelliferyl-α-L-fucopyranoside.

As can be seen from Table 7.1, two of the dogs J and W had negligible fucosidase activity consistent with diagnosis of fucosidosis. The English Springer spaniels A, G and T, had intermediate fucosidase activities typical of heterozygotes and animals E, L, P and S had fucosidase activities within the normal range.

7.3. Analysis of storage products.

Urine was available from the fucosidosis bitch, W. The urine was deionised, freeze dried and resuspended in deionised water to give a creatinine concentration of 0.9 mM. The treated urine was analysed by thin layer chromatography (TLC) on silica gel plates, which were stained with orcinol/H_2SO_4 to detect sugars. Purified storage products isolated from fucosidosis dog brain (Barker, 1987) were run alongside the urine as markers (fig 7.2.).

As can be seen from fig 7.1, bands comigrating with markers FG6, FG7, and FG8 were present suggesting that the glycoasparagines FG6, FG7 and FG8 are excreted in the urine of the fucosidosis bitch W. These storage products were also observed in the urine of human fucosidosis patients.

A band comigrating with FG2, was not found in canine fucosidosis urine but was present in the human fucosidosis urine. Bands corresponding to FG3, FG4, and FG5 were not detected in dog or human urine. A low molecular weight storage product which migrates before FG1 was present in the urine from the human fucosidosis patient but was absent from that of the fucosidosis dog.

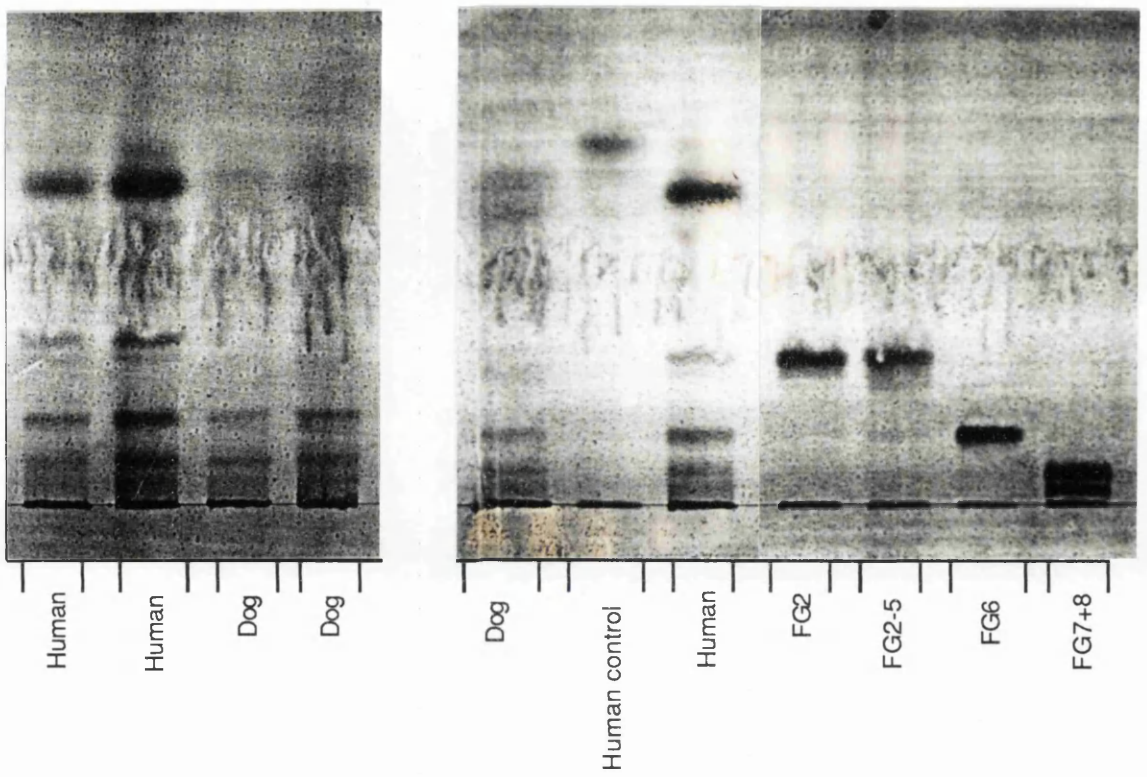


Fig 7. 1. Thin layer chromatography of urines from a fucosidosis dog and patient A.M.

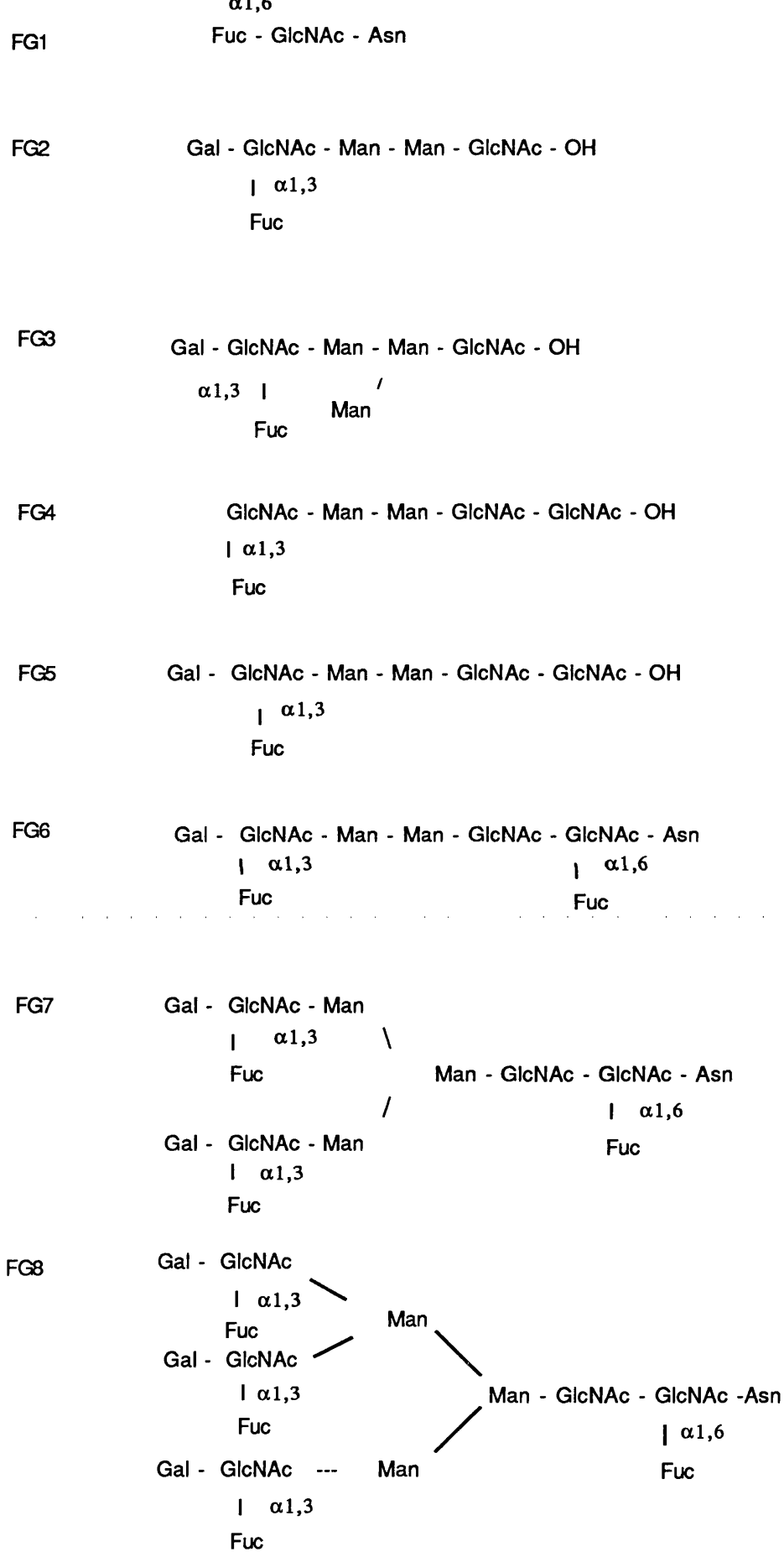


Fig 7.2 Proposed structures of fucoglycoconjugate storage products isolated from the brain of a dog affected with fucosidosis, used as markers (Barker 1987)

7.4. Messenger RNA expression.

Some information can be gained about the nature of a disease-causing mutation by investigating mRNA production. For example splice site, frameshift and nonsense mutations often result in production of unstable mRNA which is degraded, while missense mutations result in production of mRNA of normal size and quantity. To determine whether fucosidase mRNA is expressed in canine fucosidosis, total RNA was extracted from the blood of fucosidosis bitch J and control dog P and analysed by the Northern blotting technique. The RNA was subjected to agarose gel electrophoresis in the presence of formaldehyde and transferred to nylon membranes by capillary action. Since the dog fucosidase gene has not been localised or sequenced, the blot was hybridised to a ^{32}P labelled human fucosidase cDNA probe, A + B. Homology between the human and canine fucosidase gene product has been demonstrated (Barker et al., 1988b).

A single band was present in both lanes of control dog RNA whereas no bands were present in either lane of fucosidosis dog RNA (fig. 7.3). These results suggest that either no fucosidase mRNA is synthesised in fucosidosis dogs, or that the level of expression is too low to be detected by this method.

Although both types of RNA were treated in the same way, RNA from bitch J may have been degraded. To determine whether equal quantities of intact RNA had been loaded into each lane, the blot could be hybridized to a probe for a gene expressed in both types of dog, such as actin.

7.5. The canine fucosidase gene.

DNA was extracted from leukocytes of the English Springer spaniels. The DNA was digested with restriction endonucleases and subjected to agarose gel electrophoresis. The DNA was transferred to nylon membranes by the method of Southern, (1975) and the blots were hybridized to ^{32}P labelled human fucosidase cDNA probes.

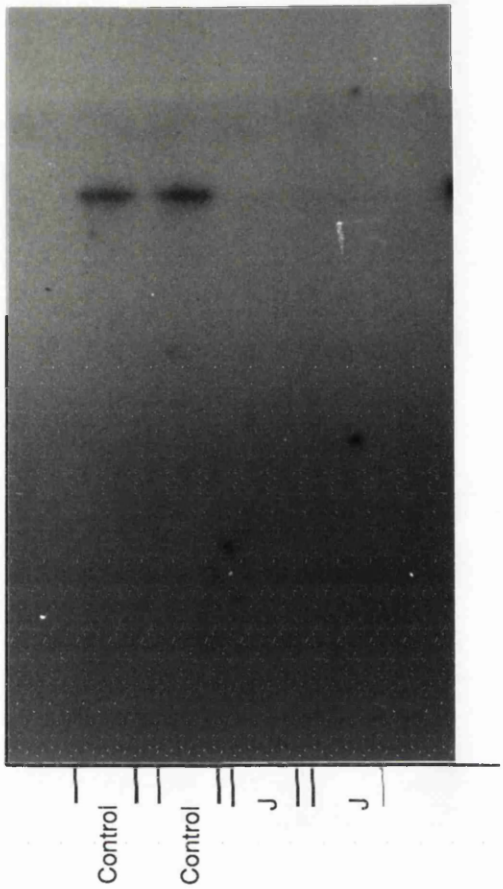


Fig 7.3. Northern blot of RNA from a control dog and a dog affected with fucosidosis hybridised to human fucosidase cDNA probe A+B.

7.5a. The normal canine fucosidase gene.

DNA from the normal dog P was digested with the restriction enzymes Pvu11, EcoR1, and BamH1 in single or double digests, with the aim of constructing a restriction map. The Southern blot was hybridized to the adjacent human cDNA probes A, B and C in succession. (Fig 7.4). An approximate restriction map was drawn (fig.7.5). The results demonstrate that the dog fucosidase gene is approximately 18 kb in length.

7.5b. The mutant canine fucosidase gene.

Southern blots were made of DNA extracted from control and fucosidosis dogs (fig 7.6). The results show that no gross gene alterations such as large insertions, deletions or rearrangements occur in the mutant gene . Fucosidosis in this dog therefore probably results from a point mutation, or a small insertion or deletion. These can only be detected by Southern blotting if they alter a restriction enzyme site. An identical pattern of DNA fragments was seen with control and fucosidosis dog DNA when digested with the enzymes Bgl11, Hinc 11, EcoR 1, Bcl1, BstE11, Pvu11 and Taq1. Invariant bands of sizes 2.1, 2.4, 3.0, and 4.5 kb were seen on Xba1 digests of affected and unaffected dog DNA. A 4.0 kb band was present in fucosidosis bitch J but not in the control dog, P (fig 7.6). The 4.0kb fragment was subsequently found to be present in a total of two control animals E and S, one heterozygote, A and the affected bitch, J. The 4.0kb was absent from two control dogs L and P and one heterozygote, G (fig 7.7). This restriction fragment length polymorphism does not, therefore, cosegregate with the disease.

7.6. Amplification of dog fucosidase gene by PCR.

Primers complementary to the human fucosidase gene were used in a PCR in an attempt to amplify part of the dog fucosidase gene. Primer 4 is complementary to the 3' end of exon 7, and primer 2 to the 5' end of the 3' non coding region. Exonic sequence primers were chosen as intronic sequences show a much greater interspecies variability. No amplification was achieved with an annealing temperature of $50^{o}C$ but amplification did occur at an

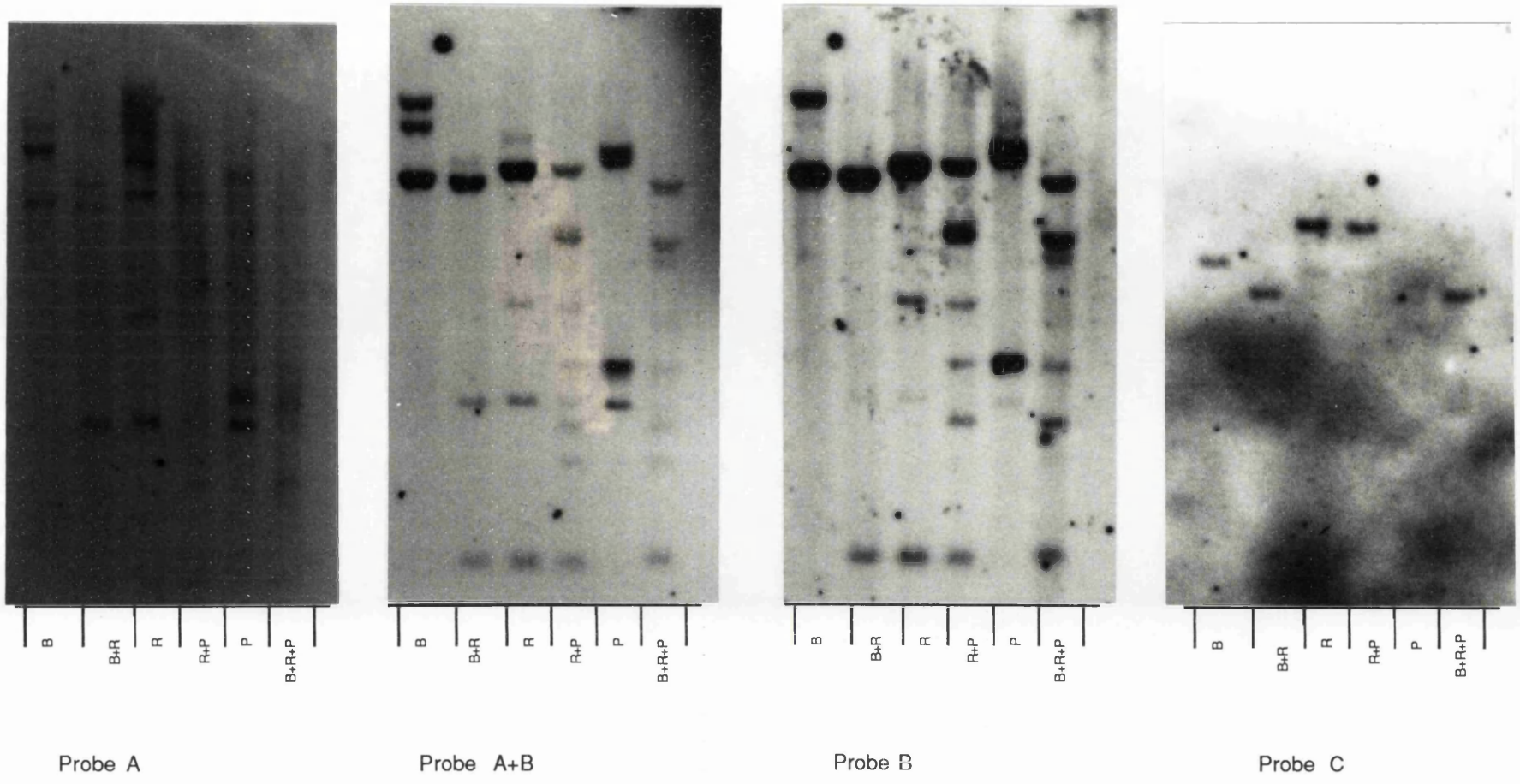


Fig 7.4. Southern blots of DNA from a control dog hybridised to adjacent human fucosidase cDNA probes.
B, BamH1; R, EcoR1; P, Pvu11.

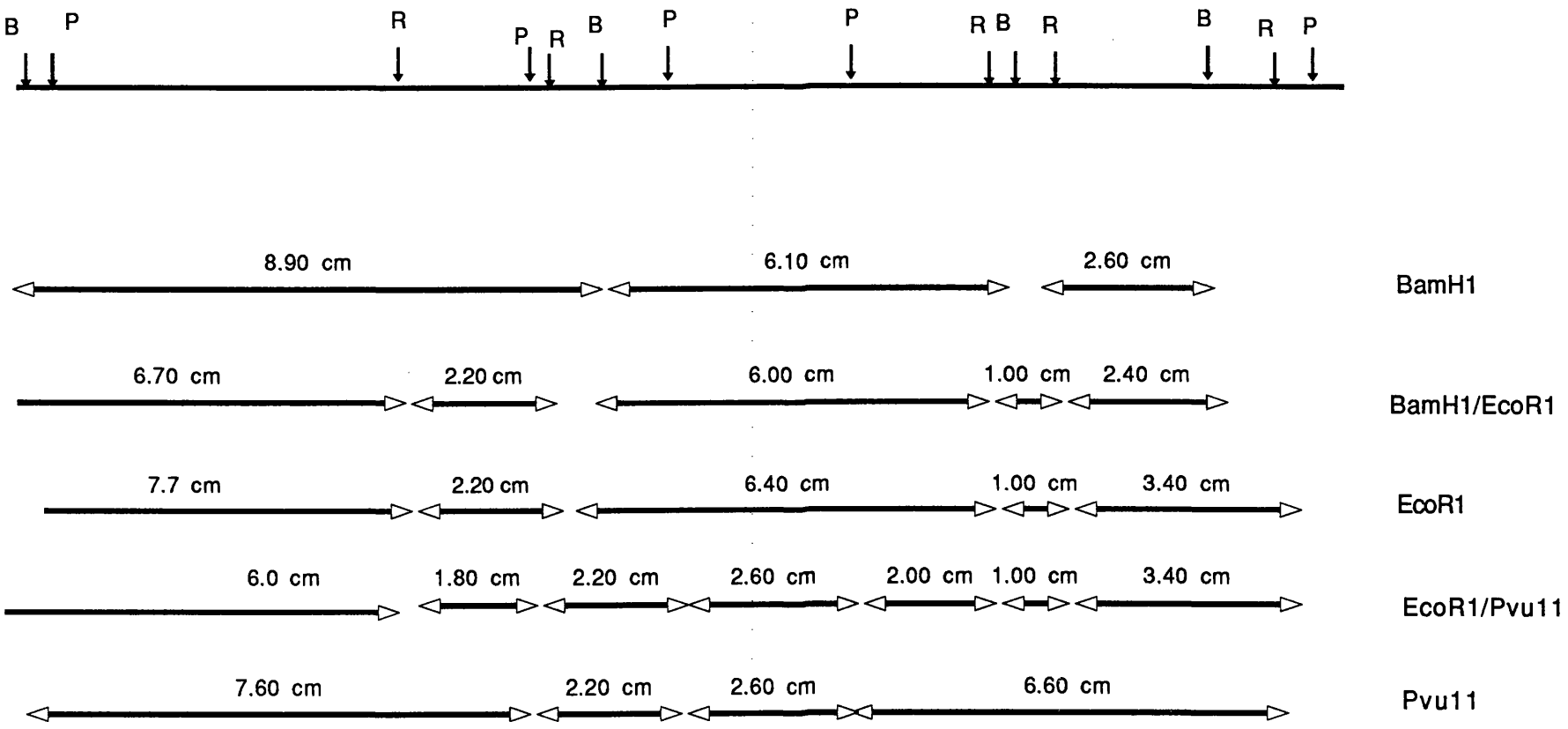


Fig 7.5. Approximate restriction map of the canine fucosidase gene.

Scale: 1 cm = ~ 1 kb; B, BamH1; R, EcoR1; P, Pvu11.

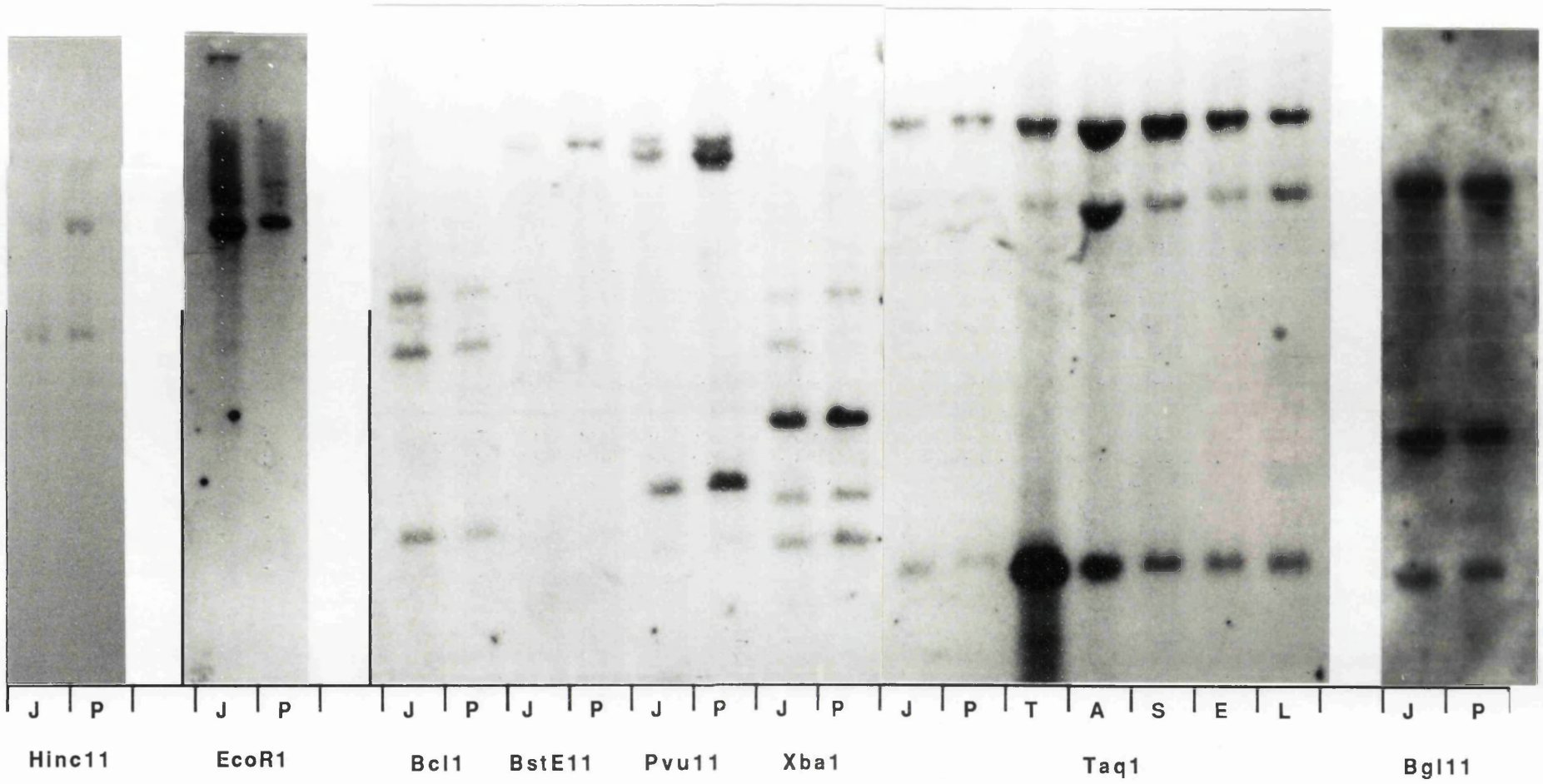


Fig 7.6. Southern blots of DNA from dogs hybridised to human fucosidase cDNA probe A+B.

J, dog affected with fucosidosis; T, A, carriers of fucosidosis;

P, S, E, L, control dogs.

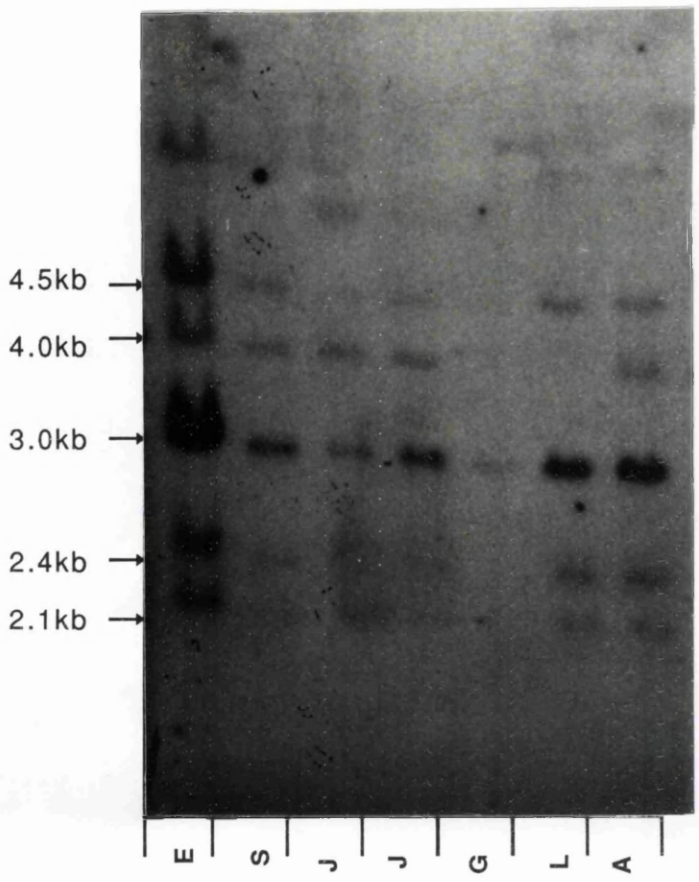


Fig 7.7. Southern blot of DNA from dogs digested with Xba1 and hybridised to fucosidase cDNA probe A+B.

J, dog J affected with fucosidosis; G, A, carriers of fucosidosis; E, S, L, control dogs.

annealing temperature of 45°C. A single band of approximately 200bp, comparable to the size of the human fragment, was seen on an agarose gel stained with ethidium bromide after amplification of DNA of three control dogs and two bands were seen with DNA from fucosidosis bitch J (fig 7.8). No bands were seen in the control lacking DNA. To determine whether these amplification products represent a part of the canine fucosidase gene or are the result of non specific amplification Southern blots of the amplification products could be hybridized to the human fucosidase cDNA probe. If the amplification products were specific for the fucosidase gene the extra band in the PCR from the fucosidosis dog may represent a disease related defect such as an insertion. However a homozygous mutation would be expected to be the cause of fucosidosis in these dogs because of the high level of interbreeding in the pedigree population.

7.7. Discussion.

Two of the animals (W and J) had no detectable α-L- fucosidase activity in plasma or leukocytes. This appears to be typical for fucosidosis dogs - leukocyte activities of less than 5% have been reported in other affected dogs (Abraham et al., 1984). Animals A, G, and T were designated as carriers of the disease and the animals E, P, L and S as normal. Three storage products which comigrated with the glycoasparagines Gal(Fuc)GlcNAcMan 2GlcNAc(Fuc)GlcNAcAsnGal(Fuc)GlcNAcMan[Gal(Fuc)GlcNAcMan]ManGlcNAc(Fuc) GlcNAcAsn and Gal(Fuc)GlcNAc[Gal(Fuc)GlcNAc]Man{Gal(Fuc)GlcNAcMan}Man GlcNAc (Fuc)GlcNAcAsn isolated from fucosidosis dog brain were present in the urine of an affected dog. FG1 (FucGlcNAcAsn) has previously been found in fucosidosis dog brain and urine, (Abraham et al., 1984) but was not detected in the urine of dog W. This was probably due to the experimental procedure. Gal(Fuc)GlcNAc Man2GlcNAc, which was present in human fucosidosis urine was absent from the dog fucosidosis urine. The canine fucosidase gene has not yet been sequenced or localised. The human and canine gene have some sequence homology since human cDNA hybridizes with the dog fucosidase gene. Cross species hybridization was used to characterise the dog gene further. Northern blotting of RNA from an affected and an unaffected dog suggested that either no mRNA is transcribed in canine fucosidosis or that it is unstable and is rapidly degraded. Lack of mRNA is consistent with the finding that fucosidosis dogs have no CRIM (Barker et al., 1988a).

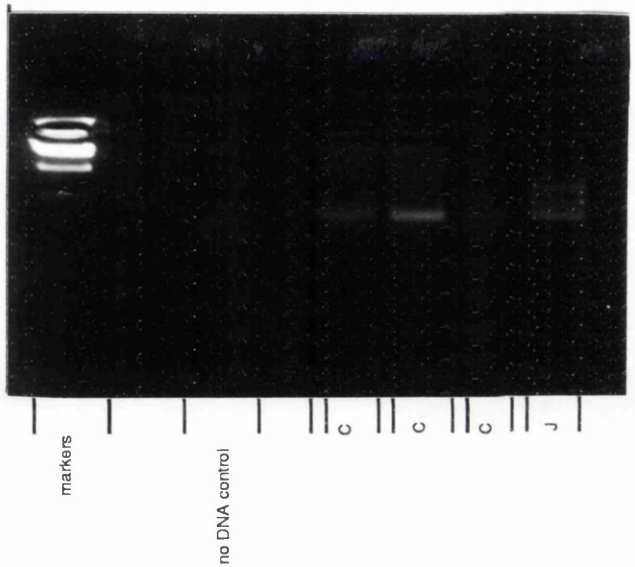


Fig 7.8. Amplified products using primers 4 and 5 of DNA from 3 different control dogs and one bitch affected with fucosidosis (J).

Restriction analysis showed that the canine fucosidase gene is approximately 18kb in length. This is shorter than the human gene which has been estimated to be 22kb. Southern blots of DNA from affected and unaffected dogs showed that the disease in these dogs was not due to any gross structural gene changes such as large deletions or insertions. The disease therefore probably results from a point mutation or small insertion or deletion which would only be detected on a Southern blot if a restriction site was fortuitously altered. One RFLP was found with Xba1, which did not cosegregate with the disease and cannot therefore represent the disease-causing mutation. If this RFLP is in strong linkage disequilibrium with the canine fucosidase gene it could be used for carrier detection in informative cases. A DNA based method of heterozygote detection would be more reliable than the enzymic methods currently used.

Homology between the human and fucosidase gene was also demonstrated by amplification by PCR of dog sequences using primers complementary to human sequences. When better characterised the product could be used as a more efficient probe for the canine fucosidosis gene.

Fucosidosis in English Springer spaniels probably results from a single mutation in the canine fucosidase gene because of the very high levels of inbreeding in the pedigree population. The human disease is, conversely, clinically very heterogenous, and probably results from many different mutations. A slightly different pattern of urinary storage products was observed between human and dog fucosidosis. However both canine and human fucosidosis result from an almost complete deficiency of the same enzyme and have a similar phenotype. Human and canine fucosidase display a high degree of homology at the DNA and protein level. Canine fucosidosis therefore provides an invaluable model system for the trials of potential enzyme or gene replacement therapies for the human disease.

CHAPTER 8

General discussion.

A mutation in the highly conserved 5'-donor splice site of intron 5 was identified in one Asian fucosidosis patient, presumably causing aberrant mRNA splicing of the fucosidase gene. The splice site mutation may result in exon skipping with a consequent in frame deletion of 201bp, or insertion of some or all of intron 5 into the mRNA if a cryptic splice site is activated. If stable mRNA were produced, Northern blotting or RTPCR could be used to determine which mechanism(s) of alternative splicing are used.

This is the third mutation to be identified in fucosidosis. The two previously identified mutations are: a nucleotide substition which creates a stop codon 120bp upstream of the normal termination site, found in 5 families of Italian, French or Cuban descent, (Willems et al., 1988), and a deletion mutation in 2 Algerian siblings (Willems et al., in press). The splice site mutation appears to be rare since it has only been found in 1 out of 22 fucosidosis patients. Fucosidosis may be similar to several other lysosomal storage diseases in which there are a few common mutations and a great number of rare mutations (for example in Gaucher disease). This is the only mutation to be found in fucosidosis patients of Asian origin to date. It would be interesting to determine whether this splice site mutation was present in other Asian fucosidosis patients. 6 Japanese patients with fucosidosis have been reported by Willems et al., (1991). A.M., also of Asian origin, did not have this mutation. As is frequently the case for autosomal recessive disorders of low incidence, homozygosity for the mutation appears to have been a result of consanguinity. The clinical phenotype of the splice site mutation is quite severe, with a relatively early onset and rapid mental and physical deterioration. However other factors appear to affect the clinical phenotype of fucosidosis patients in addition to the genotype, as shown by two siblings with fucosidosis reported by Willems et al., (1988a) who were both homozygous for the same mutation but had different clinical phenotypes. The same G -> A transition in the highly conserved +1 position of a 5'

splice site has been found in other lysosomal storage diseases including Tay Sachs and metachromatic leukodystrophy (Alki et al., 1991; Polten et al., 1991).

As is found in most fucosidosis patients, S.B., G.J., C.L. and A.M. had negligible fucosidase activity and negligible anti-fucosidase CRIM in the cases of the latter 3 patients. S.B. and A.M. also had typical storage products in their urine. In spite of this apparent homogeneity at the protein level these 4 patients are clinically heterogenous. C.L., G.J. and A.M. have different mutations from S.B.; G.J. and A.M. have a different phenotype at the RNA level and therefore probably also have different mutations. It is possible that slight differences in the residual activity, which account for the clinical heterogeneity and result from heterogeneity at the DNA/RNA level, are present but are too small to be detected by these methods. In addition the clinical phenotype of a specific mutation may be modified by unknown variables such as environmental factors or other gene products such as fucosyl transferases. Factors which affect the rate of influx of the substrate of fucosidase into the cell would also presumably affect progression of the disease. The finding of siblings with the same genotype but different phenotype supports this proposal. Some correlation between clinical phenotype and genotype has been found in other lysosomal storage disease in which there is also negligible activity. For example, Gaucher patients with the Asp370 -> Ser mutation have the non neurological type1 phenotype, whereas patients also with negligible activity but with the Leu444 -> Phe mostly have type 2 or 3 genotype. There are several possible sources of the residual α-L-fucosidase activity and CRIM detected in fucosidosis patients. They may be due to the presence of low levels of a structurally altered mutant enzyme with decreased specific activity and stability. Alternatively activity may result from inaccuracies in the assay techniques used (for example spontaneous degradation of the substrate in activity assays), secondary activities of other glycosidases, or possibly, by the action of an as yet unidentified non-lysosomal fucosidase as has been found in the rat (Nuck et al., 1987).

Patient V.O. was found to be atypical of fucosidosis patients, with fucosidase activity

increasing from low levels at 2 years of age to normal levels at 10 years with concurrent clinical improvement. This is unlikely to have resulted from an increase in gene expression or RNA stability with age since high levels of fucosidase enzyme protein were detected in fibroblasts taken at 2 years of age. Comparison of fucosidase CRIM levels in this patient at different ages would be useful. The increase in activity could be due to an increase in stability of the enzyme in the cell as the cellular environment changes with development. Instability could result, for example, from a mutation affecting a glycosylation site. The glycotype of the cells could change with age, rendering the incorrect initial glycosylation unimportant. Differences in glycosylation could be detected as changes in mobility on polyacrylamide gels. Alternatively an increase in activity could result from an increase in an activator or decrease in inhibitor of the enzyme. Mixing experiments did not indicate the presence of either (E.Young, personal comm.).

Preliminary results suggest that, as with other lysosomal enzyme genes, there are alternative transcripts of the fucosidase gene, since transcripts of two different lengths were detected by amplification of the RNA. The identity of the cDNAs could be simply checked by restriction enzyme mapping or by Southern blotting to a fucosidase cDNA probe. The mechanism of alternative splicing could then be elucidated by sequencing the amplified products individually. The smaller transcript could arise from exon skipping, or by the use of alternative 5' or 3' splice sites. Alternative splicing in the sphingomyelinase gene arises from skipping of exon 3 (Schuchman et al., 1991). This is probably due to the presence of a weak donor splice site at the exon 3/ intron 3 junction (Agt instead of the consensus Ggt). Similarly all 5' donor splice sites in the fucosidase gene have the consensus sequence Ggt with the exception of that at the exon 1/ intron 1 boundary which has the sequence Agt. It is possible that alternative splicing could arise from the occasional use of a cryptic splice site within exon 1 in preference to the weak exon1/ intron 1 donor splice site. Alternative pre mRNA splicing can be used as a device for regulating gene expression and generating isoform diversity. However, in the cases of the lysosomal enzymes sphingomyelinase, β-galactosidase and β-glucuronidase only one of the transcripts produces an active enzyme (Schuchman et al., 1991; Morreau et al., 1989; Oshima et al., 1987). The role of the other transcript(s) is unknown.

Currently fucosidosis is diagnosed routinely on the basis of enzyme activity

assays. Fucosidase activity assays are unreliable for carrier detection. This is because the control and carrier ranges of activity overlap. There is also the complication of the low plasma activity polymorphism. C.B had intermediate activity and CRIM in fibroblasts. He may therefore be misdiagnosed and be homozygous for the low activity allele. These anomalies would be avoided by the addition of DNA based assays. For example, Sa.B., a sibling of patient S.B. would have been designated a carrier on the basis of enzyme activity but homozygous normal on the basis of the Southern blotting results. Where the mutation is unknown, linkage analysis using the Pvu11 and Bgl1 RFLPs could be used in families informative for linkage. Detection of the mutation in families where the mutation is known is preferable however since it would not require material from other family members and would not require the family to be informative for linkage. The splice site mutation of patient S.B. fortuitously creates a new Taq 1 site and could therefore be readily detected by Southern blotting of DNA digested with Taq 1, or more simply by amplification of the region of the exon 5/intron 5 boundary and digestion with Taq 1.

Two methods were used to screen PCR products for the presence of mutations prior to sequencing : restriction enzyme digestion and mismatch analysis. As shown in this study restriction enzyme digestion of PCR products is a rapid simple screening technique which will detect point mutations that alter the site of the restriction enzyme used and insertion and deletions above a certain size depending on the size of fragment and the resolving power of the gel. The vast majority of mutations would be missed by this technique however.

The technique of mismatch analysis (Montandon et al., 1989), also shown in this thesis, involves the chemical modification with hydroxylamine or osmium tetroxide of unpaired bases of heteroduplexes formed between labelled wild type and mutant PCR products followed by cleavage with piperidine at the site of modification. This technique has the potential of detecting all mutations within the region amplified, but may be limited in its usefulness as a rapid screening technique because of the large number of manipulations involved.

Several other techniques have been devised to screen PCR products for mutations prior to sequence analysis. Denaturing gradient gel electrophoresis (DGGE) separates

DNA fragments according to their melting properties. The PCR products are electrophoresd through gels of an increasing concentration of denaturant. The DNA fragments remain double stranded until they reach a concentration of denaturant equivalent to the melting temperature of the lowest melting temperature domain. The partial melting of the fragment at this point in the gel sharply reduces the mobility of the fragment. Point mutations within this low melting domain will alter the melting temperature of the domain and therefore the distance along the gradient that the fragment will migrate. Addition of an approximately 40bp sequence of G/C to the product (a GC clamp,incorporated via the primers) enables all domains to be examined (Myers et al., 1985a). Differential migration can be enhanced by forming heteroduplexes between wild type and mutant DNA fragments prior to electrophoresis. This technique is relatively rapid but does not detect all mutations and does not localise any mutation found within the product.

RNase mapping involves hybridization of labelled antisense RNA probes of normal sequence to PCR products or RNA (Myers et al., 1985b). The resultant RNA-DNA or RNA-RNA duplexes are treated with RNase A which preferentially cleaves RNA at mismatched bases. Cleavage is detected by running the fragments on a polyacrylamide gel. However only approximately 50% of mutations are detected by this technique, cleavage depending on the specific mismatch and the flanking sequences.

The recently developed method of single stranded conformation polymorphism (SSCP, Orita et al., 1989) analysis is simple, rapid and has the potential of detecting all mutations within the amplified product. Denatured, labelled PCR products of mutant and wild type are electrophoresed through non-denaturing polyacrylamide gels such that the separated single strands form sequence specific conformations. Sequence differences between wild type and mutant amplified products are detected as mobility shifts caused by the conformational changes. SSCP analysis does not localise the mutation and is restricted to relatively small DNA fragments -optimal differential migration is observed with amplification products of approximately 200bp.

Direct sequencing of PCR products will detect and identify all mutations. However when a large number of samples are to be examined for the presence of mutations, the use of a rapid screening technique prior to sequencing is required.SSCP analysis is now

probably the method of choice.

Now that the intronic sequences flanking each exon of the fucosidase gene are known exons 1 to 8 from the other 5 patients could be amplified by PCR and the products screened for the presence of mutations by mismatch analysis or SSCP. The region containing the mutation from each patient could then be sequenced. Identification of the mutation in each patient would help diagnosis of further cases and carriers considerably. Correlations between genotype and phenotype may also be possible. Such correlations would enable better prognoses to be made and would help in genetic counselling and possibly in decisions about treatment strategies in the future.

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APPENDIX 1

Isolation of Human Mannose-binding protein cDNA sequence.

List of figures.

INTRODUCTION.

1.A.Animal lectins.

Lectins are nonenzymatic proteins which recognise and bind to specific carbohydrate structures. Animal lectins can be divided into several groups according to structure. The S-type, thiol dependent group includes many small (14 - 16kDa) soluble lectins which are specific for beta galactosides. The C -type, calcium dependent group includes endocytic receptors, which have a role in clearing serum glycoproteins from the circulation, (e.g. the asialoglycoprotein receptor), lectin containing cell adhesion molecules (selectins), macrophage receptors, proteoglycan core proteins, soluble proteins such as pulmonary surfactant apoprotein, and mannose binding protein. Other mammalian lectins are best known in other contexts, for example the mannose 6 phosphate receptor and serum amyloid protein.

1.B.Mannose-binding protein

1.B.1. Isolation and physical properties.

i) Human.

a). Human liver.

A human mannose binding protein (MBP) not associated with enzymatic activity was first detected during purification of α-D-mannosidase from human liver by affinity chromatography (Robinson et al., 1975). MBP was subsequently isolated from human liver by affinity chromatography on mannose-Sepharose 6B and found to have a molecular weight of approximately 1,000,000 with subunits of 30.5kDa and 28.0kDa (Wild et al., 1983).

b). Human serum.

The presence of a MBP in human serum was suggested by the observation that uptake of glycoproteins by rat hepatic sinusoidal mannose N acetyl glucosamine receptor was inhibited by a factor in human or rat serum. The inhibition of glycoprotein uptake decreased as the number of cells increased implying that the factor was a lectin which competed with the receptor for ligand binding. The lectin was purified and found to have a molecular weight of less than 35kDa (Taylor and Summerfield 1984). Serum MBP can be distinguished from

mannose specific immunoglobulins by its dependence on calcium ions for binding (Summerfield and Taylor, 1986). Two distinct calcium ion dependent MBPs of different physical properties (Table A) have been isolated from human serum (Taylor and Summerfield, 1987). Antibodies raised against MBP 1 and 2 do not cross react. MBP 1 is immunologically identical to the liver MBP.

	MBP 1	MBP 2
Native Mr	700,000	200,000
Subunit Mr	32,000	28,000
Specificity	Mannose, fucose,	Mannose, fucose GlcNAc, ManNAc, glucose
pH optimum of binding	pH7-9	pH6-7
Association constant Ka for mannan binding	$2.1 \times 10^8\ M^{-1}$	$1.3 \times 10^8\ M^{-1}$

Table A.

ii)Rat.

a).Rat liver.

MBP has been isolated from rat liver (Kawasaki et al., 1979; Townsend and Stahl, 1981; Mizuno et al., 1981; Maynard and Baenziger, 1982; Wild et al., 1983; Drickamer, 1986). Rat liver MBP binds specificaly to mannose and N-acetyl glucosamine in the core region of asparagine-linked oligosaccharides (Brownell et al., 1984) and is calcium ion dependent. Rat MBP has a subunit size of approximately 30kDa. Drickamer et al., (1986) distinguished two distinct but homologous types of rat liver MBP, designated MBP-A and MBP-C. MBP-C is the predominant form in liver and the more slowly migrating species on a polyacrylamide gel in the presence of SDS. Electrophoresis of an unreduced preparation of MBP has shown that it exists as dimers and trimers stabilised by disulphide bonds, with molecular weights of 57,000 and 78,000 respectively. Gel filtration of undenatured preparations suggests that the native MBP molecule consists of six identical subunits, consistent with either two disulphide linked trimers, or three disulphide linked dimers.

b). Rat serum.

Both types of rat MBP have been found in rat serum (Oka et al., 1988).

c). Other species.

MBP has also been isolated from rabbit liver (Kawasaki et al., 1978) and serum (Kozutsumi et al., 1980). A MBP has been isolated from chicken serum that is not dependent on calcium ions for binding (Wang et al., 1985).

1.B.2. Synthesis and secretion of MBP.

i). Rat.

Pulse labelling experiments have shown that liver MBP (MBP-C) is secreted into plasma by rat hepatocytes (Brownell et al., 1984), although at a slow rate (t1/2 = > 4hr). The lectin undergoes hydroxylation of proline and lysine, and hydroxylysine is glycosylated. Inhibition of this post-translational modification prevents secretion (Colley et al., 1987). Secretion of MBP-A by liver has been shown by Oka et al., (1988), with a turnover rate

typical of plasma proteins (1/2 life = 1.6 days). Conversely this group found that the greater part of MBP-C remained in hepatocytes and had a rapid serum turnover, suggesting that MBP-C leaks into the serum.

ii). Human MBP.

The identity of MBP-1 from serum and liver (Taylor et al., 1989) suggests that serum MBP-1 is secreted by the liver. The presence of a signal sequence and its expression as an acute phase protein also suggests that human MBP-1 is a hepatic secretory protein. The origin of MBP-2 in serum is uncertain (Taylor and Summerfield, 1987).

1.B.3.Structure of MBP.

i). Rat MBP.

a). Rat MBP-A and MBP-C.

cDNA clones encoding rat MBP-A and MBP-C were isolated from a rat liver cDNA library by screening with oligonucleotides complementary to the protein sequence (Drickamer et al., 1986) or with anti-rat liver MBP antibodies (Oka et al., 1987). The cDNA sequence of MBP-A and MBP-C were found to be 56% homologous when aligned with 4 gaps. Each polypeptide consists of three domains: 1). a cysteine-rich region involved in formation of interchain disulphide bonds; 2). a collagen-like domain of Gly-X-Y repeats with 4-hydroxy proline residues in several Y positions; 3). a carboxy-terminal carbohydrate binding domain (Drickamer et al., 1986; Oka et al., 1987). These three domains are preceded by an amino terminal hydrophobic signal sequence which is cleaved off during biosynthesis.

b). Rat MBP genomic structure.

Rat MBP cDNA was used as a probe to screen a rat liver genomic library and the genomic sequence of MBP-A was determined (Drickamer and McCreary, 1987). The rat MBP-A gene consists of four exons, with exons 3 and 4 coding for the carbohydrate recognition domain and exon 2 and part of exon 1 coding for the collagen-like domain. A pseudogene (MBP-B) sequence, 77% homologous to exon 3 and 4 of MBP-A was also isolated. MBP-C was not isolated, however.

ii). Human MBP.

a). MBP-1 cDNA and protein sequence.

Rat MBP cDNA was used as a probe to isolate human MBP cDNA sequences from a human liver cDNA library (Ezekowitz et al., 1988). Human MBP was found to have 51% homology with rat MBP-C (3 gaps), and 47% homology with MBP-A (7 gaps). Taylor et al., (1989) found a 61% and 52% homology respectively. Like rat MBP, human MBP has a structure of three domains - an amino terminal cysteine-rich domain, a collagen-like domain and a carbohydrate binding domain.

b). Genomic structure.

Human MBP cDNA or oligonucleotide probes corresponding to the cDNA sequence, have been used to isolate the genomic sequence of human MBP (Sastry et al, 1989, Taylor et al., 1989 ; see fig.A). The intron/exon structure of the gene is very similar to that of the rat MBP-A gene. Exon 1 codes for a hydrophobic signal sequence, a cysteine-rich domain which mediates interchain disuphide bonds, followed by 19 repeats of Gly-X-Y. The position of the first intron resembles the arrangement found in non fibrillar collagen. Exon 2 encodes 11 repeats of Gly-X-Y of the collagen domain, while exon 3 codes for a 'neck-like' region. The carbohydrate recognition domain, together with a 2.5kb untranslated region are encoded by exon 4.

iii). Chromosomal location.

Human MBP has been asigned to chromosome 10q11.2-q21 by in situ hybridization (Sastry et al., 1989).

1.B.4. Relationship to other animal lectins.

MBP is one of a family of calcium-dependent animal lectins which have structurally related carbohydrate recognising domains (CRD). The CRD share at least 18 invariant residues distributed throughout the domain (Drickamer et al., 1988). The group can be

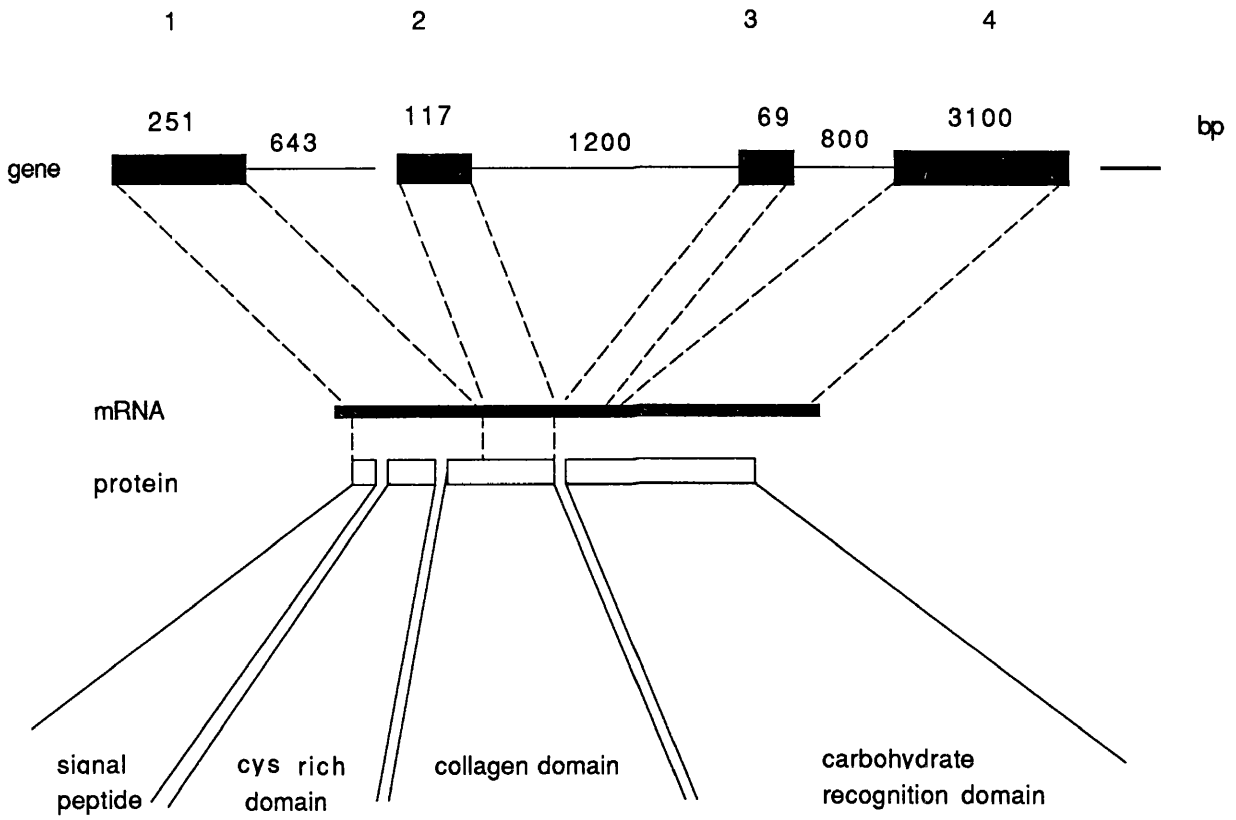


Fig.A. The human mannose-binding protein gene.

subdivided into membrane-bound proteins, in which the CRD is attached to a membrane anchor domain, and soluble proteins (such as MBP) in which the CRD is attached to a range of other domains of uncertain function. The soluble lectins, MBP and pulmonary surfactant A protein, have similar non carbohydrate recognising domains - a cysteine-rich amino terminal region and a collagen like domain. CRDs are therefore found in association with a range of other domains presumably brought about by shuffling of exons during evolution.

1.B.5. Function of MBP.

The role of MBP was first proposed to be in intracellular glycoprotein transport (Wild et al., 1983; Mori et al., 1983) or in the binding of potentially toxic mannose-terminated enzymes, such as lysosomal enzymes, which escape into the serum, prior to removal by the sinusoidal mannose receptor. A role in host defence was primarily suggested by the ability of MBP to bind yeast mannan. Several lines of evidence have given further support to this proposal:

i). MBP as an acute phase reactant.

Production of MBP appears to be regulated rather than constitutive. MBP mRNA was present in greatly increased amounts in liver from a patient who suffered major trauma prior to death. The pattern of MBP synthesis was shown to mirror synthesis of the acute phase reactants, C-reactive protein and serum amyloid A, leading to the proposal that MBP could be included as an acute phase protein (Ezekowitz et al., 1988). The sequence of the promoter region of human MBP is consistent with this conclusion. The 5'-end of the gene contains a heat shock consensus region, three glucocorticoid response elements, and a sequence with a high degree of homology to a similar sequence in serum amyloid A protein gene (Taylor et al., 1989).

ii).Inhibition of in vitro infection by human immunodeficiency virus.

Infection in vitro of CD4+ H9 lymphoblasts with HIV could be inhibited by preincubation of HIV1 with human MBP, in a dose-dependent manner. Inhibition was reversed by the addition of mannan. MBP was shown to bind to recombinant gp120 and to

HIV infected H9 cells expressing the gp120 glycoprotein. MBP probably inhibits infection by binding to the envelope glycoprotein gp120 of HIV1, which is recognised by CD4 (Ezekowitz et al., 1989).

iii). MBP as a direct opsonin.

Purified human MBP was shown to bind selectively to *Salmonella montevideo* that express mannose-rich O-polysaccharide, and not to mutants that lack mannose-rich O-polysaccharides within the lipopolysaccharide coat. MBP was also shown to enhance attachment, ingestion and killing of the bacteria by phagocytes in a dose-dependent manner (Kuhlman et al., 1989). The authors suggest that MBP acts as a direct opsonin, although a phagocyte cell surface receptor which would presumably bind MBP has not been identified. The significance of this role in vivo is unclear, however, since the levels of MBP used in these assays exceeded normal serum levels 100-fold.

iv). Complement fixation.

Rat MBP-A and human MBP-1 have been shown to activate complement (Ikeda et al., 1987). Lysis of mannan-coated sheep erythrocytes (ME) sensitised with MBP was found to be dependent on C4-containing serum and increased with increasing amounts of complement (C'), MBP, and mannan coating the erythrocytes. Lysis did not occur in the absence of C4, indicating that MBP activated lysis proceeds through the classic pathway of C' activation. In contrast to rat MBP-A, rat MBP-C did not activate complement, revealing a possible diversity of function between the two rat MBPs. MBP may either function in a similar way to C1q, interacting with C1r and C1s to promote formation of C1 esterase, or C1q may bind to ME-MBP complexes. The similarity of structure between MBP and C1q (both have a collagenous domain) supports the first proposal.

v). The association of a defect in opsonisation with low levels of MBP.

5-7% of the general population have a defect in opsonisation of S.cerevisiae for phagocytosis by normal polymorphonuclear leukocytes. The defect is associated with deposition of suboptimal amounts of C3b/C3bi (Turner et al., 1985). The opsonisation defect

is found in a series of children with unexplained infections. A significant correlation has been found between serum MBP levels and the generation of C3b opsonins. The opsonisation defect in low C3b binding serum was corrected by MBP enriched material in a dose-dependent manner (Super et al., 1990).

These results led to the proposal that a deficiency of MBP was the primary cause of the opsonic defect. This proposal was further supported by the finding of a single mutation in the MBP gene in three immunosuppressed children with the opsonic defect (Sumiya et al., 1991). The mutation (G-A) changes the 54th codon, in exon 1, from glycine to aspartic acid. This disrupts the fifth Gly-X-Y repeat and would distort the secondary structure of the collagen-like domain, possibly leading to failure of the abnormal MBP subunits to polymerise. This would explain the dominant inheritance of the disorder. Homozygotes and heterozygotes for the mutation in the families of the three affected children were found to have low MBP levels as compared to homozygous normals.

It would therefore appear that MBP has a role in host defence through activation of complement. Disease is not inevitably associated with low levels of MBP, however. This suggests that activation of complement via MBP is only an accessory system under normal circumstances. A deficiency of MBP may become pathologically important only when other defects of the immune system are present, or at 6-18 months of age when maternal antibodies have disappeared and the antibody repertoire of the infant is immature.

Rat MBP-A and human MBP 1 are both secreted as a plasma protein, activate complement and appear to have a role in host defence. Rat MBP-C, however, is found predominantly in liver and does not activate complement, suggesting that the two rat MBPs have different functions. The role of human MBP 2 may similarly differ from that of MBP 1.

6.Aim of the project.

The project was started after publication of the rat cDNA sequence, but prior to publication of the human MBP cDNA sequence. The aim of the project was to isolate cDNA clones coding for human MBP using rat MBP-C cDNA as a probe, by cross species hybridization, and to determine the cDNA sequence of human MBP. The protein structure of the human MBP could then have been derived from the sequence, thereby shedding light on possible functions of the protein. The structure could also be compared with that of rat MBP-A and C.

MATERIALS AND METHODS.

2A. Preparation of cDNA probe.

Rat MBP-C cDNA cloned into plasmid pCD in X1776, was kindly provided by Dr. K. Drickamer.

i). Isolation of plasmid DNA.

Liquid broth for X1776: 25g/l tryptone, 7.5g/l yeast extract, 20mM Tris-Cl pH 7.5, 5mM $MgCl_2$, 0.01% diaminopimelic acid, 0.004% thymidine, 0.5% glucose.

A large culture of X1774 containing the plasmid was grown in complete liquid broth for X1776. Plasmid DNA was extracted from the culture after caesium chloride gradient centrifugation, as before (see Chapter 2.4c)

ii). Excision of cDNA insert.

Plasmid DNA was digested with the restriction enzyme BamH1 to excise the insert. The digested DNA was analysed by electrophoresis in an agarose minigel containing ethidium bromide. Following identification under uv the cDNA insert was excised from the gel and the DNA was extracted from the excised gel slice with 'Geneclean'. Digestion with BamH1 and agarose gel electrophoresis was repeated to ensure minimal contamination of the probe with plasmid sequences.

The rat MBP-C cDNA was cut with Pvu1 and EcoR1 and the fragments separated by electrophoresis. The band representing the central fragment of 383bp was excised from the gel and was used as a probe for Southern blotting.

iii). Labelling of cDNA probe.

The cDNA probe was labelled with ^{32}P by random hexanucleotide labelling (see

Chapter 2.5 v).).

2.B. Screening of a λ gt11 library with the cDNA probe.

A human liver cDNA library in λgt11 was screened with the rat MBP-C cDNA probe according to the method of Huynh et al., (1986).

i). Plating out of λgt11 phage.

LBMg broth: 10g/l tryptone, 5g/l yeast extract, 10g/l NaCl, 10 mM $MgSO_4$, pH 7.5.

A single colony of Y1088 plating cells was inoculated into 3ml of LBMg broth supplemented with 0.2% (w/v) maltose and 25μg/ml ampicillin. The cultures were grown at 37°C with shaking at 200rpm for 5 hrs. 90μl of the appropriate dilution of recombinant phage was added to 100μl of Y1088 culture . The phage and Y1088 cells were mixed and the phage allowed to adsorb to the bacteria by incubating them at 37°C for 20 min. Molten 0.7% agarose in LBMg broth was then added to the phage and bacteria . The solution was inverted to mix and was then rapidly poured onto an agar plate (1.2% agar in LBMg broth, prewarmed to 37°C). The plate was swirled gently to ensure an even distribution of top agarose and bacteria. When the top agarose had solidified, the plates were inverted and incubated at 42°C overnight.

ii). Determination of the titre of the λ gt11 library or phage solutions.

SM: 5.8 g/l NaCl, 2 g/l $MgSO_4$, 50mM Tris-Cl pH 7.5, 0.01% gelatin, autoclaved.

Ten fold serial dilutions of the phage were made in SM. Each dilution was plated out with Y1088 cells as above (2 B i).). The number of plaques on each plate for each phage dilution was counted and the number of plaque-forming units (pfu) in the undiluted stock solution was estimated.

iii). Screening.

Denaturing solution: 1.5M NaCl, 0.5M NaOH

Neutralising solution: 1.5M NaCl, 0.5M Tris-Cl pH 7.2, 1mM EDTA

The appropriate number of plaque forming units of the λgt11 library or from previously isolated plaques, were plated out onto agar plates with Y1088 cells as described above (2B i).). A control plate of λgt11 with no insert was included in the secondary and tertiary screen. The plates were chilled at $4^{o}C$ for 1 hour to allow the top agarose to harden. A nylon filter (Hybond N, Amersham), cut to the same size as the plate , was placed over the plate so that the whole filter made contact with the top agarose. Five holes were made with a sterile needle, through the filter, top agarose and bottom agar in an asymmetrical pattern (to allow alignment of the filter and the plate later). The filter was left on the plate for 1.5 min, and was then peeled off. A second filter was placed on the same plate. Holes were made in the second filter in the same places as the first. The second filter was left on the plate for 2 min. Each filter was then placed in denaturing solution for 30 secs, neutralising solution for 5 min and was then rinsed in 2x SSC. The filters were allowed to air dry and then placed, DNA side down, on a uv transilluminator for 3.5min. The plates were sealed and stored at $4^{o}C$.

iv).Hybridization to the cDNA probe.

Denharts solution: 2% BSA, 2%ficoll, 2% polyvinyl pyrrolidone.

Hybridization solution: 6xSSC, 5x Denharts solution, 0.5% SDS, 0.05M sodium phosphate buffer, pH 6.8, 0.01M EDTA.

The filters were prehybridized at $65^{o}C$ overnight in hybridization solution containing 20υg/ml denatured herring sperm DNA. The filters were hybridized to ^{32}P labelled rat MBP-C cDNA probe, ($3\text{-}6x10^{5}$ cpm/ml of hybridization solution) at $65^{o}C$ overnight. The filters were then washed twice in 2xSSC at $65^{o}C$ for 15min and once in 2xSSC containing 0.1%

(w/v) SDS at 65°C for 30 min. The filters were then autoradiographed using Kodak XAR-5 film, at -70°C, for 1 to 5 days.

v). Picking of plaques.

The plate was placed over the autoradiogram and the five holes in the agar aligned with those on the autoradiogram. The plaques giving a strong signal on the autoradiogram of both the first and duplicate filter were picked out with a pasteur pipette. The small circular plug of agar containing the plaque of interest was placed in 1ml of SM with one drop of chloroform. These were left at room temperature for approximately 1hr, to allow the phage particles to diffuse out of the agar, and then stored at 4°C.

2.C. Antibody screen of λ gt11 library with antibodies against human MBP.

i). Pretreatment of the antiserum with crude lysate from a λ gt11 recombinant lysogen.

TBS: 50mM Tris-Cl pH 8.0, 150mM NaCl.

Single colonies of BNN97 (a lysogen of λgt11) were tested for temperature sensitivity at 42°C . Cells from single colonies were spotted onto two $LBMg_2$ agar plates using a sterile toothpick. One of each pair of plates was incubated at 42°C and the other at 30°C overnight. A control of uninfected Y1088 cells was included on each pair of plates. BNN97 colonies that grew at 30°C but not 42°C were assumed to be lysogens and were used in lysate formation.

One colony of BNN97 lysogen was inoculated into 100ml of LB pH 7.5 . The culture was incubated at 30°C with shaking until it had reached an absorbance of 0.89 (measured at 600nm). The culture was incubated at 42°C for 20 mins. IPTG was then added to 10mM and the culture was incubated at 37°C for a further hour. The cells were harvested by

centrifugation in a Beckman JA-14 rotor at 5,000rpm for 5mins at 24°C. The pellet was resuspended in PBS. The resuspended cells were frozen in a dry ice-methanol bath. The cells were then thawed and sonicated.

A nylon filter (Hybond N, Amersham) was incubated in the BNN97 lysate for one hour on a roller. The filter was rinsed in TBS for 10mins and then incubated in TBS containing 20% (v/v) fetal calf serum for 15 mins. The filter was transferred to solution of TBS with 20% (v/v) fetal calf serum which also contained ten fold diluted anti human MBP1 serum and was incubated overnight on a roller.

ii). Screening.

Small cultures of Y1090 were mixed with appropriately diluted recombinant phage and plated out with top agarose as before (see 2B i).). The plates were incubated at 42°C for 4 hours. A nylon filter, cut to the same size as the plate was soaked in a 10mM solution of IPTG for 1 min and then allowed to dry in the air. The filter was then placed on the plate so that it was in contact with the top agarose. The plates were incubated at 37°C overnight. Five holes were made in the filter and agar for later alignment of the plate and filter. The filters were peeled off the plates and rinsed in TBS. The filters were then incubated in TBS containing 20% (v/v) fetal calf serum for 30mins at room temperature with gentle shaking. The filters were then transferred to a sterile beaker containing TBS, with 20% fetal calf serum, and anti human MBP1 serum. The antibody solution had been pretreated with BNN97-bound filters to remove any components which would bind E.coli or λ gt11 antigens (see 2C i).). The antibody solution was diluted 100 fold. The filters were incubated in this solution for 3 hours with shaking. The filters were then washed successively in: TBS for 10 mins, TBS containing 0.1% NP-40 for 10mins and TBS again for 10 mins. Finally they were rinsed in TBS containing 20% (v/v) fetal calf serum. They were then transferred to TBS containing 20% (v/v) fetal calf serum and 2,000 fold diluted goat anti-rabbit IgG conjugated horseradish peroxidase and incubated for 1 hour. After washing in TBS, TBS containing 0.1% NP-40 and TBS for 10min periods each the filters were incubated in horseradish peroxidase colour

development solution (Biorad) for 30min. This solution was prepared according to the manufacturers instructions.

2.D. Extraction of λ gt11 DNA.

i). The plate lysate method.

Recombinant phage were plated out with Y1088 as before (see 2B i).), to achieve a high density of plaques. The plates were incubated at 42°C overnight. 5ml of SM was put onto each plate and the phage in the plaques were eluted out by gently shaking the plate for two hours at room temperature. The SM was taken off the plate with a pipette. 100μl of chloroform was added, and the solutions stored at 4°C. The eluted phage were then spun at 3,000rpm for 10min in an MSE bench centrifuge to remove the bacterial debris. The supernatant was recovered and RNAase and DNAase were added to a final concentration of 1μg/ml. The mixture was incubated at 37°C for 30mins. An equal volume of the following solution was then added: 20% (w/v) polyethylene glycol and 2M NaCl in SM. The solution was incubated at 0°C for 1hour and then spun at 10,000g for 20mins to recover the precipitated phage particles. The pellet was resuspended in SM by vortexing and the suspension spun at 8000g for 2min to remove any remaining debris. SDS and EDTA was added to the supernatant to a concentration of 0.01% (w/v) and 5mM respectively and the solution was incubated at 68°C for 15min. The solution was then extracted with phenol, phenol/chloroform and chloroform. An equal volume of isopropanol was added to the final aqueous phase and the tubes were left at -70°C for 20mins. The tubes were allowed to thaw and then centrifuged in an Eppendorf centrifuge for 15min. The resultant pellet of DNA was washed with 70% ethanol, allowed to dry and then resuspended in TE.(pH7.8). The bacteriophage DNA was stored at -20°C.

ii). Large scale preparation.

A 250ml culture of Y1088 was grown overnight. The optical density of the culture at 600nm was measured to estimate the concentration of cells

($8x10^8$ cells/ml have an optical density of approximately 1.0). Approximately $6x10^9$ cells were spun for 10mins at 4000g and then resuspended in 1.5ml of SM. Approximately 10^6 pfu of recombinant phage were added and the bacteria and phage were vortexed to mix. They were then incubated at 37°C for 20mins. The phage and bacteria were next added to 500ml of prewarmed LB. The flask was incubated at 37°C with shaking overnight. 10ml chloroform was added to each 500ml culture. The cultures were shaken at 37°C for a further 30min. The cultures were then cooled to room temperature and DNAase an RNAase were added to 1μg/ml. The solutions were left at room temperature for 30 mins. 29.2g of NaCl was added to each 500ml culture to give a final concentration of 1M. The solution was swirled to mix and was then left on ice for one hour. The cultures were spun at 4°C for 10min at 11,000g to remove debris. Polyethylene glycol (PEG 8000) was added to the supernatant to a final concentration of 10%. Once dissolved, the solution was left on ice for 1 hour to allow the phage particles to form a precipitate. The solution was then spun at 11,000g for 10mins at 4°C. The phage pellet was resuspended in 8ml SM. The cell debris and PEG were removed from the phage suspension by adding an equal volume of chloroform and vortexing for 30secs, followed by centrifugation at 3000g for 15mins at 4°C. The aqueous phase was recovered and 0.5g per ml of CsCl was added. The solution was layered onto a CsCl step gradient. The step gradient was made by layering three solutions of decreasing density one on top of the other (1.70g/ml, 1.50g/ml, 1.45g/ml). The tubes were spun in a Beckman SW28 rotor at 22,000rpm for 2hrs at 4°C. The layer of phage particles was extracted from the tube with a needle and syringe. The phage particles were put in an ultracentrifuge tube which was then filled with 1.5g/ml CsCl solution. The tubes were spun at 38,000rpm in a Ti50 rotor for 24hrs at 4°C. The band of phage particles was extracted from the tube with a needle and syringe. The CsCl was removed by dialysis overnight at 4°C against: 50mM Tris-Cl buffer pH8.0 containing 10mM $MgCl_2$ and 10mM NaCl. EDTA to a final concentration of 20mM, pronase to a final concentration of 0.5mg/ml and SDS to a final concentration of 0.5% (w/v)

were then added. The solution was mixed by inverting the tube and was then incubated at 37^{o}C for 1 hour. The solution was extracted with phenol, phenol/chloroform (50:50) and chloroform and dialysed against TE overnight at 4^{o}C.

The phage DNA was stored at -20^{o}C.

2.E. Southern blotting.

The DNA extracted from the λgt11 recombinants was digested with EcoR1 and subjected to agarose gel electrophoresis. The DNA was transferred to a nylon filter (Hybond N, Amersham) by Southern blotting (see Chapter 2.5d). The filter was hybridized to ^{32}P labelled rat MBP-C cDNA (see Chapter 2.5dv.).

2.F. Amplification of λgt11 insert by the polymerase chain reaction.

Primers complementary to the arms of the β-galactosidase gene in λgt11, either side of the cloning site, were constructed:

Primer A: 5' TCC TGG AGC CCG TCA GTA TC
Primer B: 5' ACT GGT AAT GGT AGC GAC CG

The reaction mixes were set up as before (see Chapter 2.5f.) with 50pmol of primer A and primer B. The reactions were denatured for 10 mins at 95^{o}C and then cooled to 50^{o}C, at which temperature the Taq polymerase was added. This was followed by 30 cycles of 72^{o}C for 2.5mins, 95^{o}C for 1min, and 50^{o}C for 1.5 min; with an extension step of 72^{o}C for 10 min at the end.

The amplification products were analysed on an agarose minigel as before. When more than one product was present, the bands representing the products were excised from the gel separately. The gel slice was put in 100 μl of TE and was frozen and thawed twice. 1μl of this solution was used in subsequent PCRs.

2.G. Direct sequencing.

The amplified product of primers A and B was sequenced directly as before (see Chapter 2.5h.). Primer A was used in the sequencing reaction.

RESULTS.

3a. Introduction.

Since the amino acid sequence of human MBP had not been determined, it was decided to use a rat MBP-C cDNA as a probe to screen a human liver cDNA library in λgt11 with the aim of isolating human MBP cDNA. Prior to screening the library the rat probe was tested on human genomic DNA, to establish the feasibility of this approach.

3b. Cross species hybridization.

To confirm that the rat MBP cDNA would hybridize to human DNA, and could therefore be used as a probe to screen the library, a Southern blot of human DNA was hybridized to the rat probe. Distinct bands were seen, indicating that cross species hybridization between rat and human MBP DNA occurred (fig B).

3.c. Primary screen.

Approximately 3.5×10^5 plaque forming units (pfu) of the λgt11 library, plated with E.coli Y1088, were initially screened, (3.5×10^4 per 137mm plate). Duplicate filters were made of each plate. The filters were hybridized to the ^{32}P labelled rat MBP-C cDNA probe.

Several spots were seen on the autoradiograms of each filter. 12 plaques corresponding to strongly positive signals on the autoradiograms of both the primary and duplicate filter were picked. (fig C).

3.d. Secondary screen.

Each plaque isolated from the primary screen was titred. Approximately 2,000 pfu of each isolated plaque was subsequently rescreened with the same probe. More positive plaques were seen on the primary screen than expected, possibly due to non specific hybridization of the probe to λgt11 sequences. In an attempt to remove all vector DNA from the MBP-C cDNA probe, it was digested twice with BamH1, and was separated twice from vector DNA

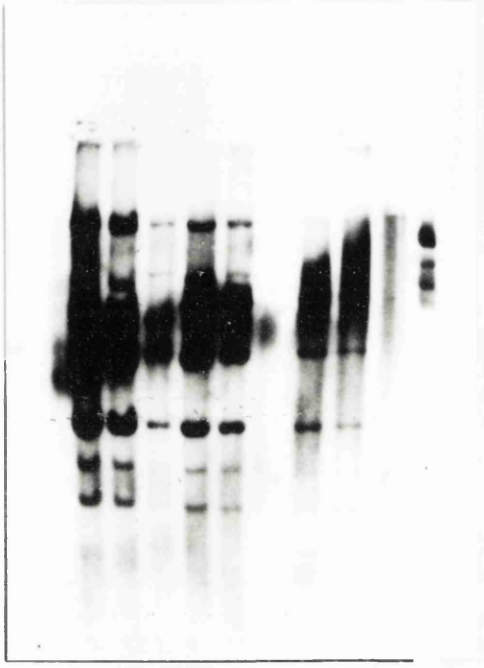


Fig B. Southern blot of control human DNA digested with Pvu11, hybridized to rat MBP-C cDNA probe.

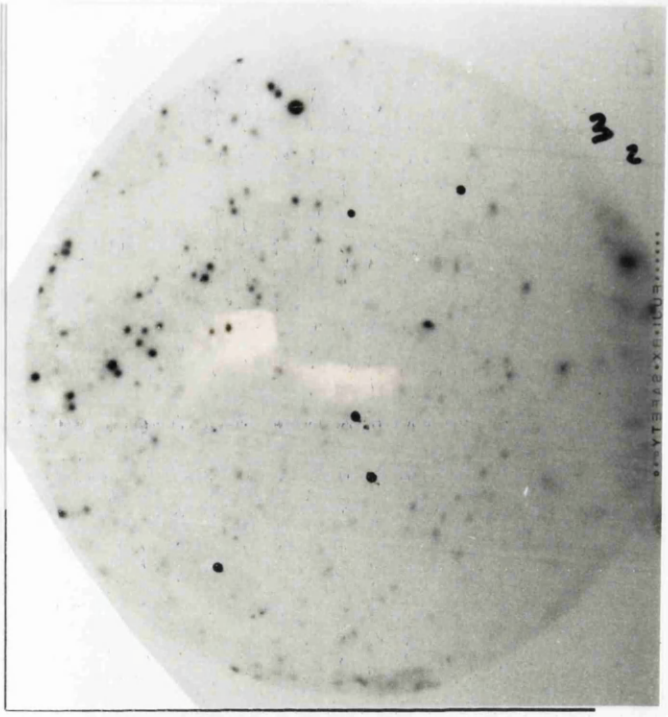


Fig C. Representative filter from the primary screen.

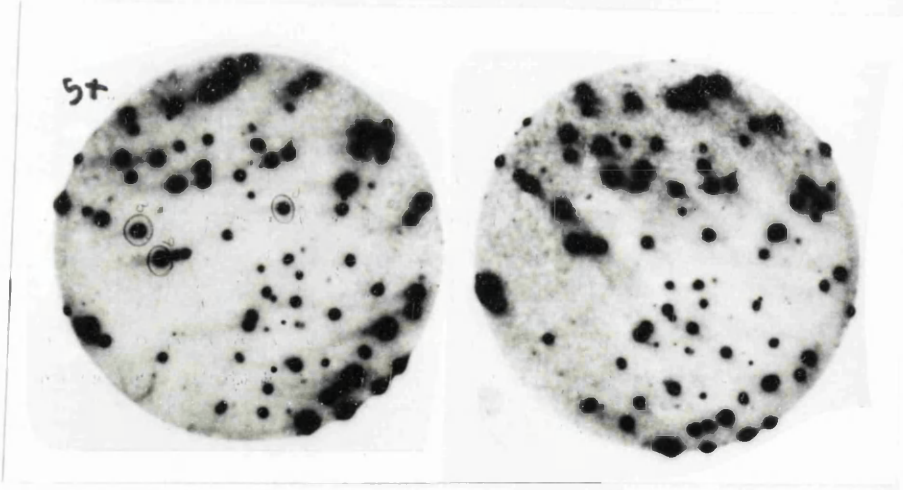


Fig D. Representative duplicate filters from the secondary screen.

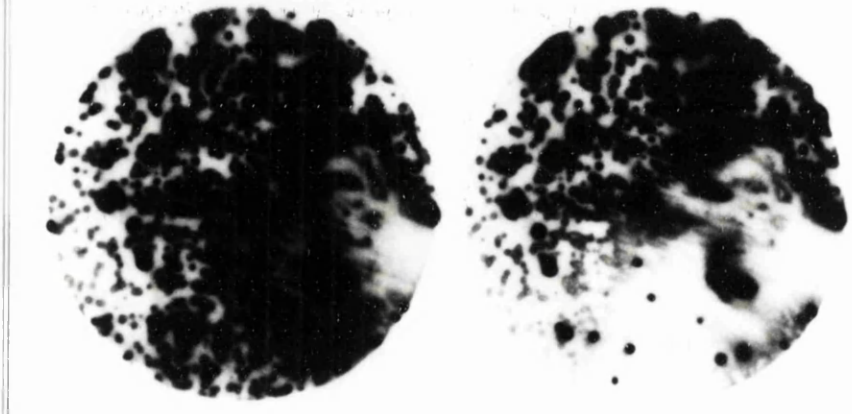


Fig E. Representative duplicate filters from the tertiary screen.

by agarose gel electrophoresis, prior to labelling. A control of λgt11 with no insert was included, and duplicate filters were made of each plate (fig D).

A greatly increased number of positive plaques were present on the secondary than the primary screen. The probe did not hybridize to filters lifted from the plate of λgt11 with no insert, implying that the positive signals from the recombinant plates were due to the cDNA inserts and not to vector sequences. 16 plaques were picked from the secondary screen, each of which hybridized strongly to the probe, both on the first and duplicate filters.

3.e. Tertiary screen.

Five plaques from the secondary cDNA screen were titred, and 1000pfu of each were plated with Y1088. A control plate of λgt11 with no insert was included. Individual plaques corresponding to the darkest signals were carefully picked (fig E).

3.f. The antibody screen.

Positive clones selected with the rat MBP-C cDNA probe, from the secondary screen, were also screened with anti-human MBP-1 antibody with the aim of confirming their identity as MBP cDNA. Recombinant phage were plated out with E.Coli Y1090. LacZ directed gene expression was then derepressed by addition of IPTG and the lacZ fusion protein blotted onto a nylon filter. The filter was incubated with anti-human MBP-1 serum and binding was detected with a horse radish peroxidase conjugated second antibody.

Prior to the antibody screen, the antiserum was treated with nylon filters to which a lysate of BNN97 had been bound. The purpose of this step was to remove antibodies from the antiserum which bind antigens normally produced by E.coli or λgt11 and thereby to reduce the background reaction.

E.coli Y1090 contains a lac repressor which inhibits lacZ gene expression until it is derepressed with IPTG. Plaque formation was initiated without lacZ expression to ensure that

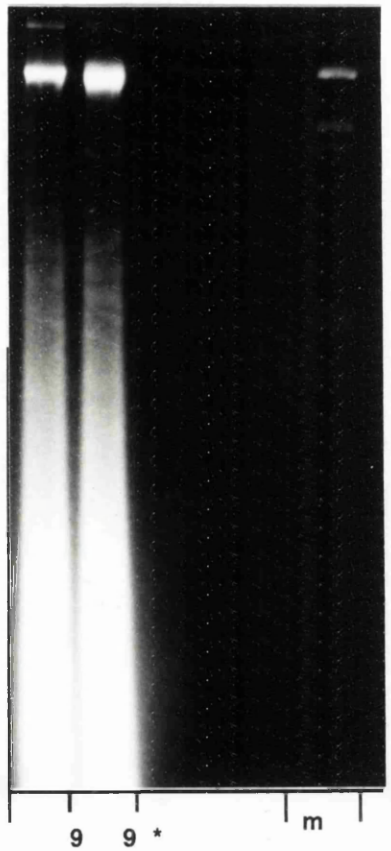
fusion proteins toxic to the host do not inhibit growth of the clones. Y1090 also has a deficiency of lon protease which increases the stability of the β-galactosidase fusion protein.

A few positive plaques were seen on some of the filters. No positive signals were present on filters from the control plate of λgt11 with no insert. The low number of positive signals on the filters suggests that a number of different clones were present. The absence of positive signals on an antibody screen does not necessarily mean that the desired clone is not present. A given DNA fragment can be inserted in two different orientations, and in one of the three possible reading frames; only one of which is correct for expression of the protein.

3.g. Southern blotting of recombinant DNA.

DNA was extracted from the recombinant clones isolated by the tertiary cDNA screen. The DNA was digested with EcoR1 to excise the insert and was then subjected to agarose gel electrophoresis. The cDNA inserts were not visible on the agarose gel, possibly because the insert (7kb maximum) is small compared to the size of the vector (43.7kb) and so the quantity of insert DNA to vector DNA loaded will be low. One band of insert was visible following blotting of the gel and hybridization to the rat MBP-C cDNA probe (fig F). Inserts were not seen on the blots of the other clones however, possibly because they had run off the gel or they were not cut out of the vector with EcoR1.

To confirm that the insert was being cut out of the vector, another EcoR1 digest was carried out. One third of the reaction product was run on an 0.3% agarose gel stained with ethidium bromide. At this low percentage of agarose the two arms of λgt11 of 19.5kb and 24.2kb produced by digestion with EcoR1 could be clearly resolved, demonstrating that cleavage had occurred (fig G). The remaining two thirds of the product of restriction enzyme cleavage was loaded onto a 2% agarose gel (fig H), which was blotted and hybridized to the rat MBP-C cDNA probe. The ends of the cDNA probe were removed by digesting the probe with Pvu1 and EcoR1 to ensure no plasmid sequences remained. The probe hybridized to uncut λgt11, but not to EcoR1 digested DNA, implying that the probe hybridized to the insert rather than the vector.



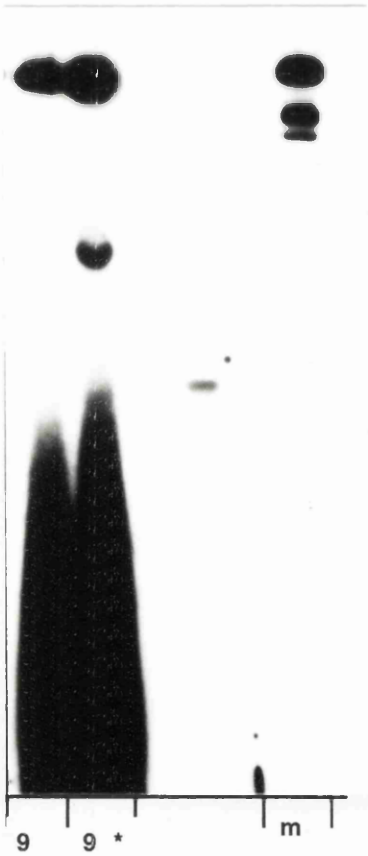


Fig.F.i). Agarose gel stained with ethidium bromide of λ gt11 clone 9 digested with EcoR1.

ii). Southern blot of the adjacent agarose gel hybridised to rat cDNA MBP-C probe.

* digested with EcoR1.

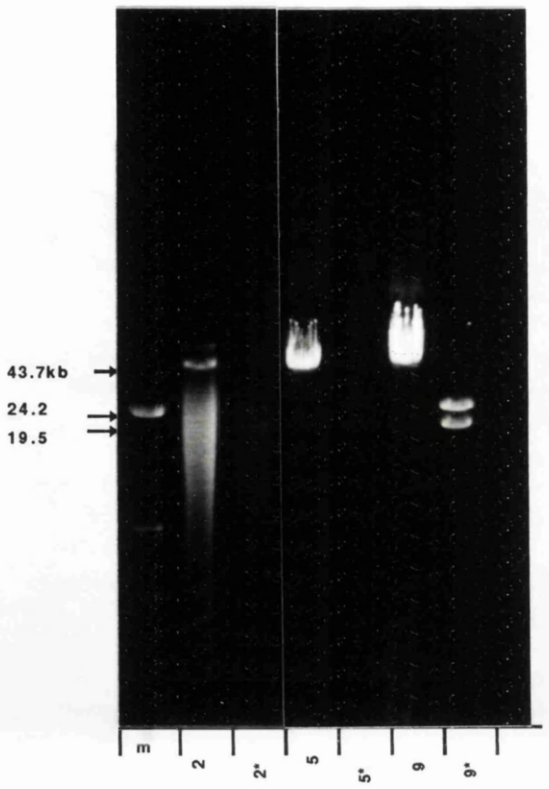
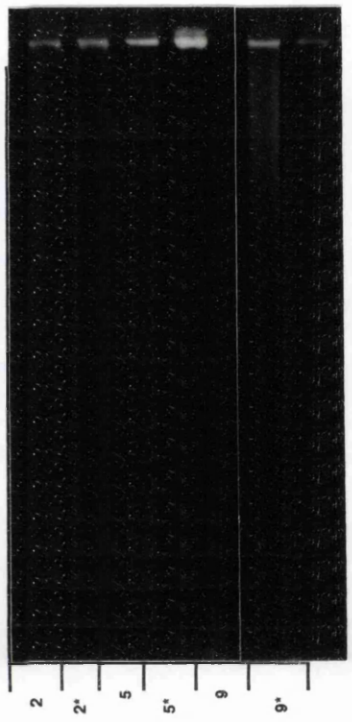


Fig. G.i). 0.3% agarose gel stained with ethidium bromide of λgt11 clones digested with EcoR1.

* digested with EcoR1.



ii). 2% agarose gel of the same samples as fig. G.i).

* digested with EcoR1.

iii). Southern blot of agarose gel in fig. G.ii). hybridized to rat MBP-C cDNA probe.

* digested with EcoR1.

3.h. Amplification and sequencing of the insert.

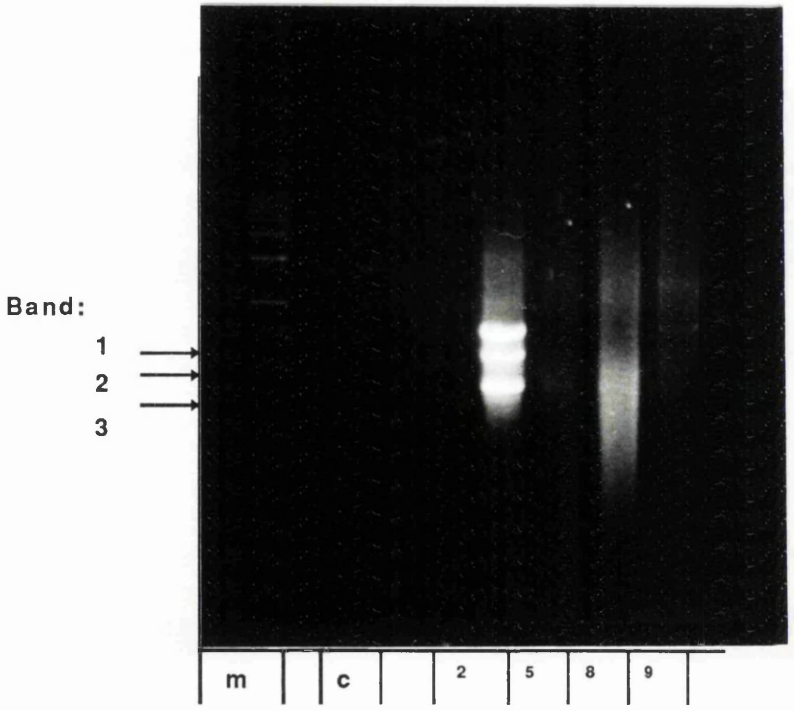
Oligonucleotide primers complementary to the β-galactosidase gene of λ gt11, either side of the cloning site, were constructed. The primers were used to amplify the insert by the polymerase chain reaction (PCR). DNA extracted from four of the clones isolated by the tertiary screen were amplified. For each clone a band of approximately 60bp was seen on agarose gels stained with ethidium bromide (fig H). This may represent amplification across the cloning site of λgt11 with no insert. Bands representing much larger amplification products were also seen. These were separated on an agarose gel by electrophoresis and then cut out of the gel individually. The DNA was eluted out of the gel slice and the DNA reamplified to give single bands (fig H). These amplified products were then sequenced directly. As can be seen from fig.I the lower part of the sequence is that of the cloning site of λgt11 with no insert. The sequence includes the sequence of the 5' end primer (primer A) which was used for sequencing and amplification, except for the first base. This is unexpected as the sequencing reaction involves addition of ^{35}S dATP to the primer where thymidine residues are present in the complementary chain. The upper part of the sequence is a novel sequence:

3'CGCTGGTCTTGCCGACCGCACGCCGCATCAGCGCTGACGAGCAACACCAGC
AGAG 5'

4. Discussion.

Three rounds of screening with a rat MBP-cDNA probe led to the isolation of four single clones of λgt11 which hybridized strongly to the rat probe. Two of these, picked after the second nucleic acid screen, also gave positive signals with an antibody screen, although at that stage more than one clone was present. This implies that at least two of the clones expressed a fusion protein to which anti human MBP 1 would bind.

Some of the amplified products of the clones were of the size expected if parental λgt11 clones lacking an insert were present. Three other larger amplified products were identified, possibly of insert DNA. These products were sequenced directly using one of the



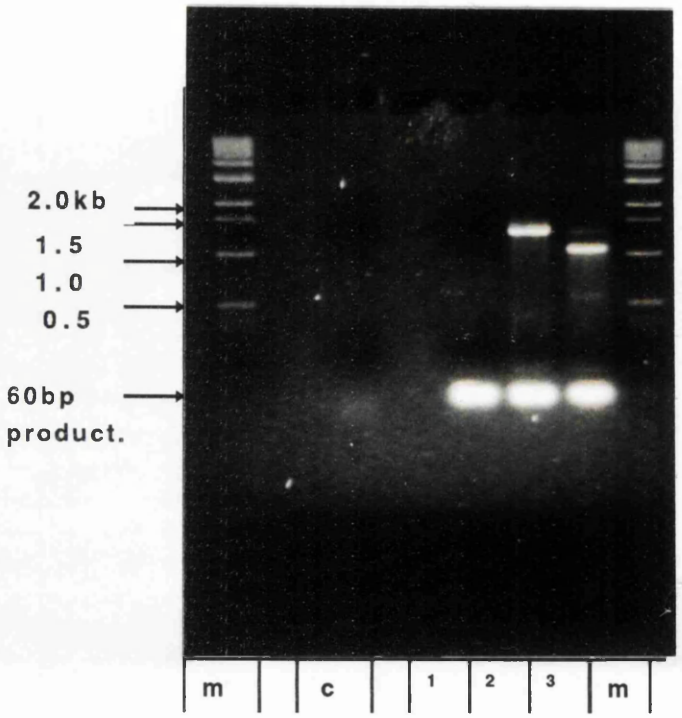


Fig. H.i). Agarose minigel stained with ethidium bromide of the amplified products of primers A and B, complementary to λgt11 arms. The bands were excised and reamplified separately.

c = control amplification with no DNA.

m = 1 kb ladder markers.

2,5,8,9 = clone number.

Fig H.ii). Reamplified products from adjacent gel.

c = control amplification with no DNA.

m = 1 kb ladder markers.

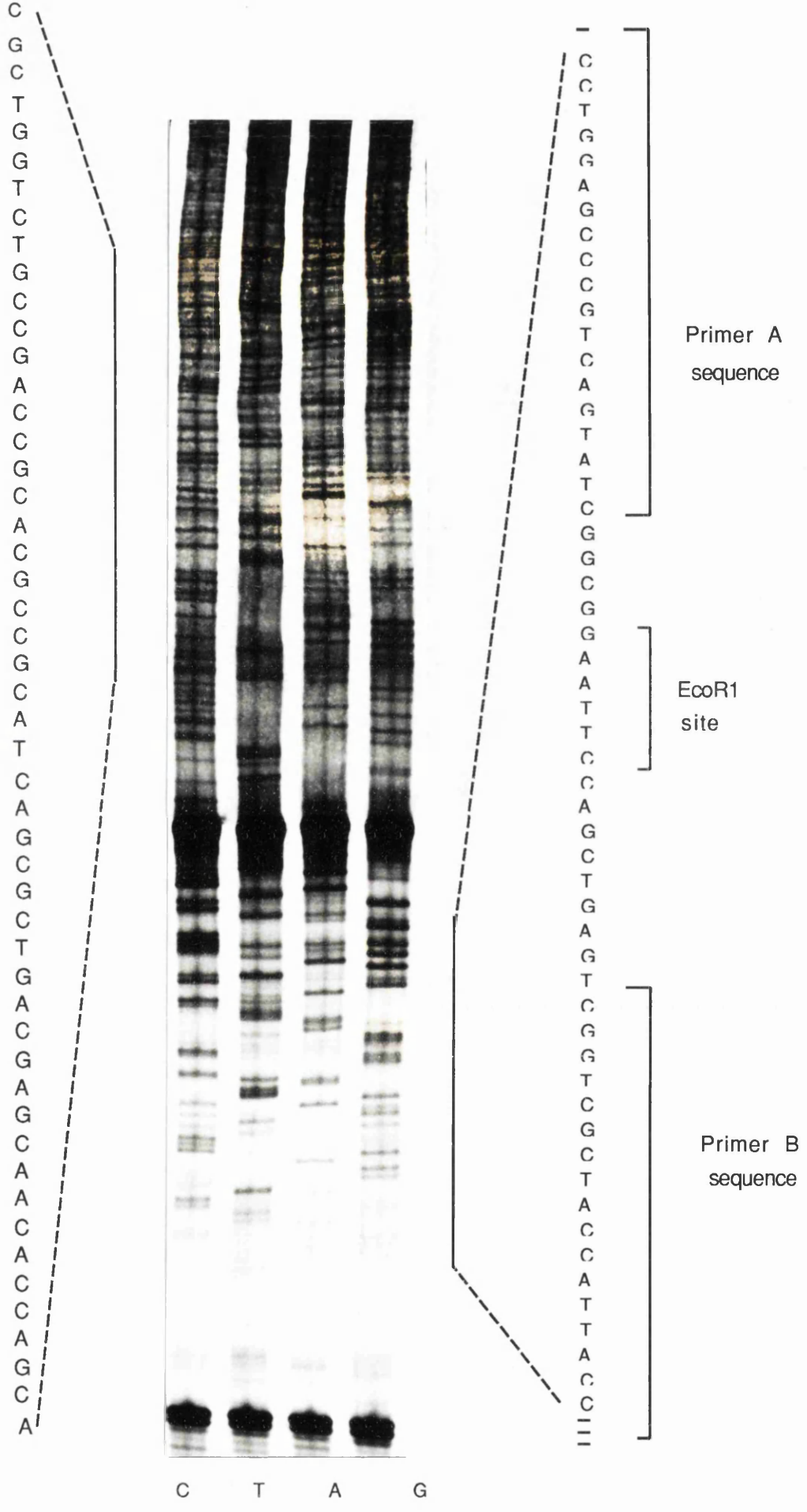


Fig.I. Sequence of λgt11 clone from fig.H. ii).

primers for amplification.The sequencing gel consisted of two regions: 1) The λ gt11 cloning site with no insert, including the sequence of the sequencing primer, 2)a novel sequence, possibly of the insert. To generate the cloning site sequence the sequencing primer may have annealed to the vector DNA to an unidentified region of homology upstream of the cloning site.

It therefore appears that the isolated positive clones were contaminated with parental phage lacking an insert. Unlike the vector λgt10 (whose cloning site is within the phage repressor gene, cI) the expression vector λgt11 does not have an efficient selection method to allow only recombinant phage to grow. High ratios of recombinant to non-recombinant phage are often therefore established during construction of the library. λgt11 with no insert would probably amplify preferentially in a mixture of parental and recombinant phage due to the size difference of product. Subcloning of the cDNA inserts into M13 and sequencing would alleviate these problems.

The upper region of the sequencing gel is a novel sequence which may be the sequence of a cDNA insert or part of the vector. An identical sequence was not found on EMBL or Genbank nucleic acid data bases (Geneworks, release 3, July 1991). Alignment of the novel sequence to the Lac Z gene of the λgt11 vector and human and rat mannose-binding protein is shown in fig. J.

Two MBPs have been isolated from rat liver and serum MBP-A and MBP-C, which are 56 % homologous. Rat MBP-A appears to have an analagous function to human MBP-1, since both activate complement and are secreted by liver into plasma. MBP-C may therefore be the rat counterpart of human MBP-2. It is tempting to speculate that sequences isolated by screening the library with rat MBP-C may be part of human MBP-2.

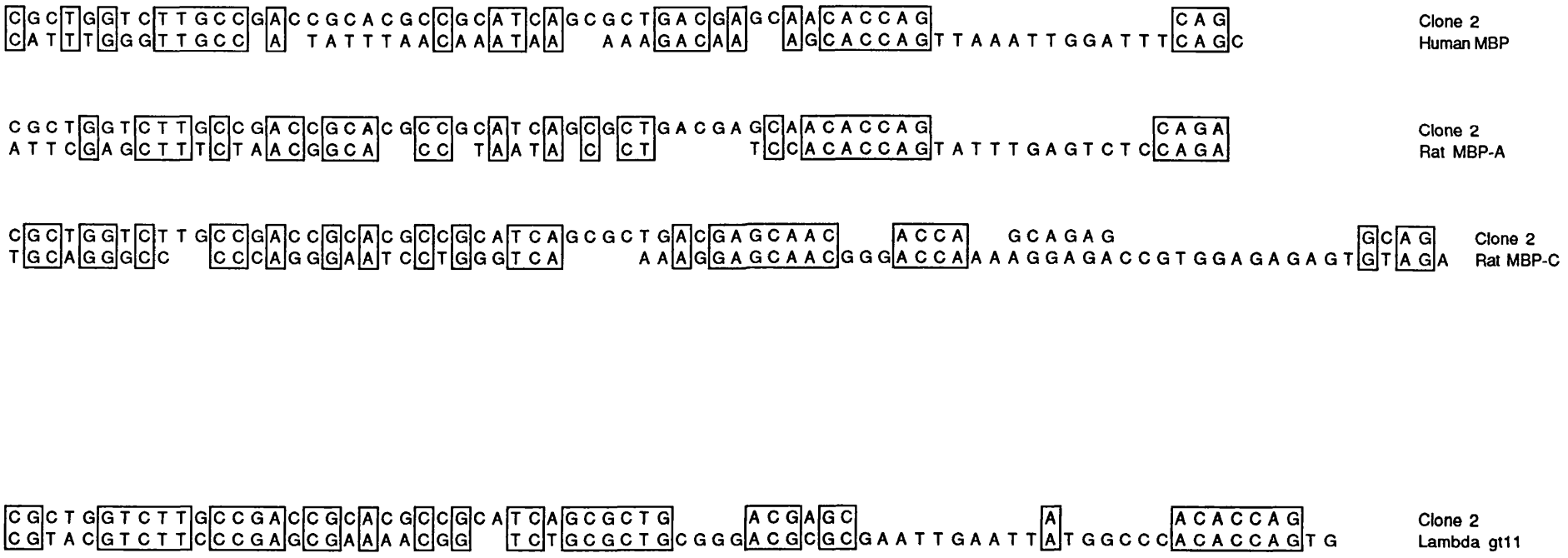


Fig.J. Alignment of the novel sequence (from clone 2) with rat and human MBP and lambda gt11 sequences.

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Appendix 2.

Materials.

Reagent.	Manufacturer.
Acetic acid (glacial)	Fisons.
Acrylamide	BDH
Agarose	BRL
Alcohols:	
Absolute ethanol	Hayman Ltd.
Other alcohols	Fisons
Ammonium acetate	Sigma
Ammonium persulphate	Bio Rad
Antibiotics	BRL
Antibodies:	
Alkaline-phosphatase conjugated goat anti-rat IgG	Sigma
Bacto agar	Difco
Bacto tryptone	Difco
Bacto yeast extract	Difco
beta-mercaptoethanol	Sigma
Bromophenol blue	BDH
BSA	Sigma
Caesium chloride	BRL
Chloroform	Fisons
Coomassie protein assay reagent G-250 based	Pierce Ltd

Diethanolamine	Sigma
Diethylamine	Aldrich
Diethylpyrocarbonate (DEPC)	Sigma
Dimethyl dichloro silane (2%)	BDH
Dimethyl sulphoxide (DMSO)	Sigma
Dithiothreitol	Pharmacia
Duolite MB 6113 mixed resin	BDH
EDTA	Sigma
Ethidium bromide	Sigma
Ficoll	Sigma
Formaldehyde	BDH
Formamide	Sigma
Geneclean kit	Bio 101 inc.
Glutamine	BRL
Glycine	Sigma
Glycogen (mussel)	Boehringer Mannheim
Guanidine	Sigma
Hams medium	BRL
HBIOI cells	BRL
HEPES	Sigma
Hexanucleotides	Pharmacia
HPTLC-Alufolien, Kieselgel 60	Merck
Hybond N	Amersham
Hydroxylamine	Aldrich
Klenow	Boeringer Mannheim

Lysozyme	Sigma
Magnesium chloride	BDH
Markers (DNA)	BRL
N,N'-methylene bis acrylamide	BDH
4-methyl umberiferyl glycosides	Koch-light laboratories
Mineral oil	Sigma
3[N-Morpholino] propanesulphonic acid (MOPS)	Sigma
p-nitrophenyl phosphate	Sigma
Nonidet NP40	Sigma
Nucleotides	Pharmacia
Nusieve GTG agarose	FMC bioproducts
Orcinol	BDH
Osmium tetroxide	Aldrich
Phenol	Life Tech.
Piperidine	Aldrich
Polyethylene glycol	Sigma
Polyvinylpyrrolidone (PVP)	Sigma
Protein A (Staph. Aureus)	Sigma
Proteinase K	BCL
Pyridine	Aldrich
Radionucleotides	Amersham
Restriction endonucleases	BRL
Reverse transcriptase	Promega
RNasin	Promega

Salmon sperm DNA	Sigma
Sephadex G-50	Pharmacia
Sequenase kit	USB
Sera:	
Foetal calf	BRL
Newborn calf	BRL
Sodium acetate	Sigma
Sodium azide	BDH
Sodium chloride	BDH
Sodium citrate	BDH
Sodium dodecyl sulphate (SDS)	Sigma
Sodium sarkosyl	Sigma
Spermidine	Sigma
Sucrose	BDH
Taq DNA polymerase	Promega
TEMED	Bio Rad
T4 polynucleotide kinase	BRL
Tris base	Sigma
Trypsin	BRL
Urea	Sigma
Vanadyl ribonucleoside complexes	Sigma
Xylene cyanol	BDH

Appendix 3.
List of abbreviations.

Asn	Asparagine
ASA	Aryl sulphatase A
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
CRIM	Cross reacting immunological material
dNTP	Deoxynucleotide triphosphate
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulphoxide
E.coli	Escherichia coli
EDTA	Ethylenediaminetetra aceticacid
ELISA	Enzyme-linked immunoabsorbent assay
FCS	Fetal calf serum
Fuc	Fucose
Gal	Galactose
Glc	Glucose
GlcNAc	N-Acetyl glucosamine
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethane sulphonic acid)
kb	kilobase
kDa	kilo Dalton
LAP	Lysosomal acid phosphatase

Man	Mannose
MBP	Mannose- Binding Protein
MOPS	3-(N-morpholino)propanesulphonic acid
4MU	4 methyl umbelliferyl-α-L-fucoside
NP40	Non idet p 40
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PIC	Polymorphism information content
PFU	Plaque forming unit
PNP	*p*-nitrophenyl-α-L-fucoside
RFLP	Restriction fragment length polymorphism
RTPCR	Reverse transcription-Polymerase chain reaction
SDS	Sodium dodecyl sulphate
SSCP	Single stranded conformation polymorphism
TEMED	N,N,N',N'-tetramethylethylenediamine
TLC	Thin layer chromatography
Tris	Tris (hydroxymethyl) amino methane
U.V.	Ultra violet