

A 5' splice site mutation in fucosidosis

Magali Williamson, Helen Cragg, Judith Grant, Keith Kretz, John O'Brien, Patrick J Willems, Elisabeth Young, Bryan Winchester

Abstract

Fucosidosis is a rare, autosomal recessive, lysosomal storage disease, resulting from a deficiency of the enzyme α -fucosidase (EC 3.2.1.51). It is characterised clinically by progressive mental and motor deterioration, growth retardation, coarse facies, and often recurrent infections, but the course of the disease is variable. The gene encoding lysosomal α -fucosidase has been mapped to the short arm of chromosome 1 at position 1p34.1-36.1 and has been called FUCA1. Two mutations causing disease have been described previously, a C→T change in exon 8 giving rise to a premature, in frame TAA stop codon, and a deletion of at least two exons from the 3' end of the gene.

In this paper we present evidence that a homozygous G→A transition in the first position of the 5' splice site of intron 5 of FUCA1 is the disease causing mutation in a 9 year old child of distantly related parents. A new banding pattern was detected in the patient by Southern blotting of genomic DNA using *TaqI* restriction and a cDNA FUCA1 probe. The patient was homozygous for this pattern. Three sibs with α -fucosidase activity below the normal reference range and both parents were heterozygous. This pattern was not detected in 26 other fucosidosis patients and has not been found in any controls. The mutation was localised by a combination of restriction mapping using different cDNA probes, single stranded conformational polymorphism analysis of exons and flanking regions amplified by the polymerase chain reaction, and by direct sequencing of the amplified sequence. In view of the nature of the mutation, its cosegregation with the disease mutation and its absence in controls, it is probable that this 5' splice site mutation causes fucosidosis in this child.

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Fucosidosis is a rare, autosomal recessive, lysosomal storage disorder caused by a deficiency of the enzyme α -L-fucosidase (EC 3.2.1.51).¹ The deficiency of α -L-fucosidase results in the accumulation in lysosomes of tissues and excretion in urine of oligosaccharides, glycoasparagines, and glycolipids containing fucose. A review of 77 patients with fucosidosis has recently been published.² The clinical picture of fucosidosis is heterogeneous but patients generally show progressive mental and motor deterioration, growth retardation,

coarse facies, recurrent infections, dysostosis multiplex, angiokeratoma corporis diffusum, visceromegaly, and seizures.²

The gene encoding human α -fucosidase has been mapped to the short arm of chromosome 1 in 1p34.1-36.1 and has been called FUCA1.^{3,4} The cDNA sequence is 2053 bp long with an open reading frame of 461 amino acids, a proposed signal peptide of 22 amino acids, and four potential glycosylation sites.⁵ The α -fucosidase gene is composed of eight exons spanning 23 kb of DNA and the genomic structure, including all the intron/exon boundaries, has been published.⁶

To date, two mutations that result in fucosidosis have been described. A single base change, C→T, which obliterates an *EcoRI* site in the cDNA has been identified in seven patients from five unrelated sibships.^{2,7,8} This transition gives rise to a premature, in frame TAA stop codon encoding a truncated fucosidase protein. The second mutation, which is responsible for fucosidosis in two Algerian sibs, is a deletion of at least two exons at the 3' end of the α -fucosidase structural gene.² The existence of at least two other mutations is indicated by haplotype analysis based on restriction fragment length polymorphisms.² Extensive Southern blot analysis in our and other laboratories has not shown any other major gene deletions or insertions in FUCA1 of patients, thus indicating that the majority of cases of fucosidosis are most likely caused by point mutations.

We report here on the characterisation of a point mutation in the 5' splice site of exon 5 of a new patient with fucosidosis. We have used Southern blot analysis and single strand conformation polymorphism (SSCP) analysis of PCR amplified sequences to localise the mutation and direct sequencing to characterise the nature of the mutation.

Materials and methods

CLINICAL DETAILS

The proband (SB) was born after a normal term delivery in Zambia to distantly related Asian parents. Birth weight was 3800 g. There are five normal older sibs. Her early milestones were normal. She smiled at 8 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, started mouthing and casting objects, and her language skills deteriorated to grunts and pointing. Her gait became progressively more unsteady. The family moved to the UK in 1989. At 6½ years she had no words but waved 'bye bye' and pointed to indicate her needs.

Division of
Biochemistry and
Metabolism, Institute
of Child Health,
London WC1N 1EH.
M Williamson
H Cragg
E Young
B Winchester

Department of
Neurosciences,
University of
California, San Diego,
La Jolla, CA 92093,
USA.
K Kretz
J O'Brien

Department of
Medical Genetics,
University of
Antwerp—UIA,
Universiteitsplein 1,
B-2610 Antwerp,
Belgium.
P J Willems

Department of Child
Health, Leicester
Royal Infirmary,
Leicester LE1 5WW.
J Grant

Correspondence to
Dr Winchester.

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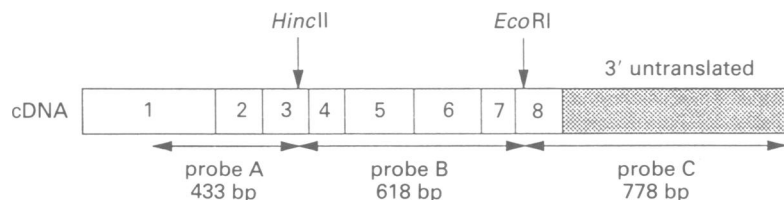


Figure 1 α -fucosidase cDNA probes. The numbers correspond to the different exons of FUCA1.⁶

She could finger feed a biscuit but could not use a spoon. She walked on a broad 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 hirsutism. There was no hepatosplenomegaly or skin lesions. Breathing was noisy owing to upper airway obstruction and a Harrison's sulcus was visible. She had a thin muscle bulk with normal tone and reflexes. She continuously mouthed and cast objects and had no constructive play. There was no corneal clouding and vision was normal. Distraction testing indicated hearing thresholds raised at 50 dB bilaterally with flat tympanograms. Currently, aged 9 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 to SB.

ASSAY OF LEUCOCYTE α -FUCOSIDASE

Leucocytes were isolated from heparinised blood as described previously.⁹ α -fucosidase activity was measured in sonicated cell extracts by incubating 5 μ l of the extract with 95 μ l of H₂O and 100 μ l of 1.6 mmol/l 4-methylumbelliferyl α -L-fucopyranoside in citrate-phosphate buffer, pH 4.5 (Melford Laboratories, Haverhill, Suffolk, UK) for 30 minutes at 37°C. The reaction was stopped by the addition of 2.3 ml of 0.25 mol/l glycine/NaOH, pH 10.4 and the fluorescence of the released 4-methylumbelliferone was read at 440 nm using an excitation wavelength of 365 nm. One unit of activity equals a rate of hydrolysis of 1 nmol per hour per mg of protein. Protein was measured using the method of Lowry *et al*¹⁰ with human serum albumin as the standard.

α -FUCOSIDASE cDNA PROBES

Human fucosidase cDNA,¹¹ encoding the major part of FUCA1, was digested with *Hin*-

cII or *EcoRI* to generate fragments A, B, and C (fig 1). The fragments were separated by electrophoresis in agarose and the DNA extracted from the agarose using 'GeneClean' (BIO 101). The probes were generated by labelling the fragments by the random hexanucleotide procedure¹² using [α -³²P] dCTP (Amersham International).

SOUTHERN BLOT ANALYSIS

Genomic DNA was isolated from peripheral blood leucocytes by using the guanidinium chloride extraction procedure.¹³ DNA (10 μ g) from members of the index family, 25 controls, and 26 other unrelated fucosidosis patients was digested with *TaqI*, *BglII*, or *EcoRI* restriction endonuclease according to the manufacturer's instructions. The resulting fragments were separated on a 0.8 to 1% agarose gel and transferred to a nylon membrane by standard procedures.¹⁴ The blots were then hybridised with the radiolabelled α -fucosidase cDNA probes. Prehybridisation and hybridisations were carried out in 10 \times Denhardt's solution, containing 4 \times SSC, 50 μ g/ml denatured salmon sperm DNA, and 1% (w/v) sodium dodecyl sulphate (SDS) for 16 hours at 65°C. Filters were washed twice for 20 minutes at room temperature in 3 \times SSC containing 0.1% (w/v) SDS. This was followed by a higher stringency wash at 65°C for 30 minutes in 0.5 \times SSC containing 0.1% (w/v) SDS. The membrane was wrapped in 'cling film' and placed in an x ray cassette with Kodak Xeromat film for 24 hours to a week.

POLYMERASE CHAIN REACTION (PCR) ANALYSIS

Exons 5 and 6 and their flanking regions were amplified by PCR using the primers in the table and fig 2. The primers were synthesised on an Applied Biosystems 381A DNA synthesiser, using DMT-biotin-C6-PA for primer 5 (Cambridge Research Biochemicals Ltd, Northwich, Cheshire, UK), according to the manufacturer's instructions. Genomic DNA (500 ng) in a total volume of 100 μ l was denatured at 94°C for 10 minutes, after which 2.5 units of *Taq* polymerase (Promega) were added and the mixture was held at 54°C for five minutes. This was followed by 30 cycles of one minute at 72°C, one minute at 94°C, and 30 seconds at 54°C. The amplified DNA was analysed by electrophoresis on a 1.5% agarose gel and visualised by staining with ethidium bromide; 20 μ l of amplified product was digested with *TaqI* according to the manufacturer's instructions (NBL, Framlington, Northumberland, UK) and analysed by electrophoresis on a 2% agarose gel followed by staining with ethidium bromide.

SINGLE STRANDED CONFORMATIONAL POLYMORPHISM (SSCP)¹⁵ ANALYSIS

Exons 5 and 6 and the exon 5/intron 5 boundary of the α -fucosidase gene were amplified by PCR using the primers and conditions already described. The reaction was carried out in a

Structures of primers used for PCR and sequencing.

Primer No	Sequence	Exon/intron
1	5' TAA GCA TGA TGC CAG GCT TG	Intron 5
2	5' AGG AGA TAC CAG TTC CGG AT	Intron 6
3	5' GAT GAG GTG GTA GTA AAT GA	Exon 5
4	5' GAA ATG ATT TCA GAT TCT TC	Exon 5
5*	5' CCT GGG GCT ATC GTC GTG AC	Exon 5
6	5' TAT AAA ATA ATA CAT ACT GCA TGT TA	Intron 5

* Biotinylated at the 5' end.

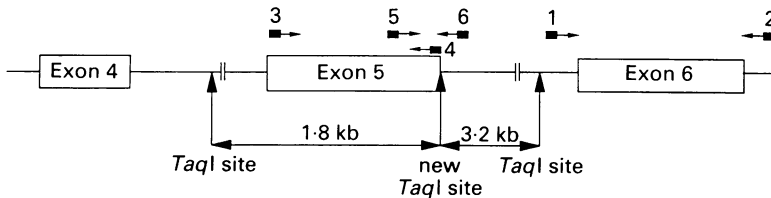


Figure 2 Primers used in the location of the new *TaqI* mutation.

volume of 50 μ l using 500 ng of DNA, 25 pmol of each primer, 0.2 mmol/l dCTP, 2 mmol/l dATP, dGTP, dTTP, 0.1 μ l of 32 P dCTP. The amplified products were analysed by electrophoresis of 10 μ l on a 1.5% agarose gel, followed by ethidium bromide staining. Five μ l of the PCR product was diluted with 40 μ l of 10 mmol/l EDTA/0.1% SDS and 5 μ l of this was further diluted with 5 μ l of gel loading buffer (80% formamide/89 mmol/l Tris/2 mmol/l EDTA/89 mmol/l boric acid, pH 8.0, with bromophenol blue and xylene cyanol added as indicator dyes). The DNA samples were denatured by heating at 95°C for three minutes and then placed on ice. Aliquots of 2.5 μ l were loaded onto a non-denaturing 6% polyacrylamide gel (Protogel Lablogic) containing 5% glycerol. Electrophoresis was carried out at 4°C for 12 hours at 360 volts and then the gels were dried and exposed to x ray film without intensifying screen.

SEQUENCING

The exon 5/intron 5 boundary was amplified by PCR using primers 5 and 6 (table, fig 2) under the conditions already described. The reaction was carried out in a volume of 100 μ l containing 5 pmol of each primer, 2 mmol/l dNTPs, reaction buffer (Promega), and 500 ng of patient DNA. Fifty μ l of the amplified product was mixed with 30 μ l of magnetic Dynal M-280 streptavidin beads (Dynal, UK) and incubated at room temperature for five minutes and then placed in a magnetic rack. The beads with the bound double stranded amplified product were then incubated with 0.15 mol/l NaOH for five minutes to produce single stranded molecules. The biotinylated single strand attached to the beads and the unbiotinylated single strand in the supernatant were separated. Two volumes of absolute etha-

anol and 1/10 volume of 3 mol/l sodium acetate, pH 5.6, were added to the supernatant which was stored at -20°C for 12 hours and then centrifuged in a bench centrifuge for 15 minutes to recover the precipitated DNA. Water (7 μ l) was added to the pellet and the DNA sequenced using the Sequenase kit (United States Biochemical Corporation) according to the manufacturer's protocol and 5 pmol of the 5' biotinylated primer 5. The samples were heated to 85°C for two minutes before 2 μ l was applied to a 6% denaturing polyacrylamide gel (Accugel, Lablogic) and electrophoresis was carried out at 55 W on a BRL (model 2) sequencing apparatus for two to two and a half hours. The gel was fixed in 10% methanol and 12% acetic acid for 30 minutes, then dried and exposed to x ray film at -70°C.

Results

α -L-FUCOSIDASE ACTIVITY

The α -L-fucosidase activity in the leucocytes from members of the family is shown in fig 3. No activity could be detected in the patient (SB) consistent with the diagnosis of fucosidosis. Both parents had levels of activity at the lower end of the control reference range, and three of the five sibs had activity lower than normal. Another sib, SaB, had similar activity to her parents and one sib, DB, had normal activity.

SOUTHERN BLOT ANALYSIS

Genomic DNA was extracted from white blood cells of SB, digested with four different restriction endonucleases, and analysed by Southern blotting using probes A, A + B, B, and C. No abnormalities were seen after digestion with *Bgl*II, *Eco*RI, or *Pvu*II. The normal pattern obtained with *Eco*RI excludes both the mutation obliterating an *Eco*RI site in exon 8⁷⁸ and the deletion at the 3' end, which is characterised by an extra junction fragment of 12 kb.²

SB was homozygous for the 12.0 kb allele of the *Bgl*II RFLP and the 7.0 kb allele of the *Pvu*II RFLP (haplotype 1-1).¹⁶ Almost complete linkage disequilibrium has been found between these two polymorphisms in North American Caucasians but a significantly lower disequilibrium was found in Belgian Caucasians.² The two sibs with a 3' deletion of *FUCA1*² also had haplotype 1-1.

When *TaqI* digested genomic DNA from SB was analysed using probe B, the 5.0 kb band which had been observed in all controls disappeared and an extra 1.8 kb band was observed (fig 4). SB appears to be homozygous for a new *TaqI* restriction site, which has not been detected in any control samples or other patients by us or others.² The parents showed both the normal 5.0 kb and the aberrant 1.8 kb band. The new *TaqI* restriction site cosegregated with the fucosidosis allele in the family of SB (fig 3). The abnormal *TaqI* restriction pattern was not present in 26 additional unrelated fucosidosis patients, nor in the control population (n = 25). The parents and three of

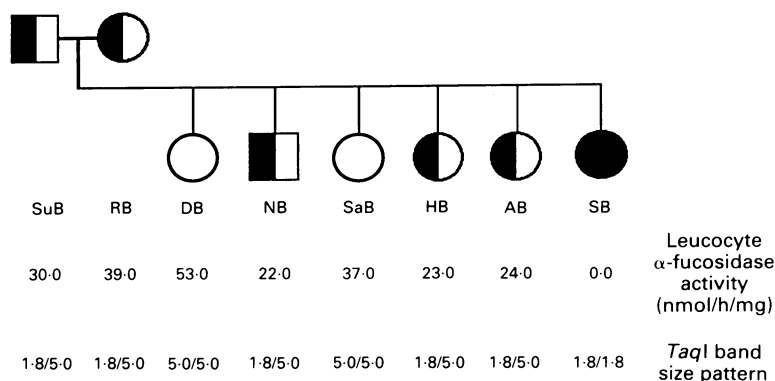


Figure 3 Family pedigree with α -fucosidase enzyme activity as compared to *TaqI* Southern blot pattern of *FUCA1*. Normal reference range of α -fucosidase in leucocytes 30–189 nmol/h/mg.

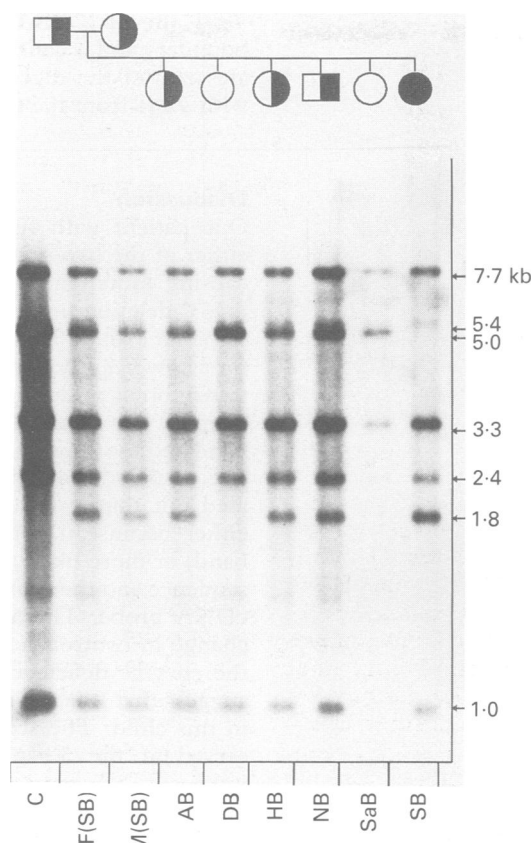


Figure 4 Southern blot analysis of *TaqI* digested DNA from the family of the patient hybridised to α -fucosidase cDNA probe B. F, father; M, mother; SB, patient; AB, DB, HB, NB, and SaB, sibs of patient; C, control.

the patient's sibs were heterozygous for this *TaqI* restriction pattern. The sib DB, who had the highest activity, and the sib SaB, whose activity was similar to that of her parents, were not heterozygous for the *TaqI* polymorphism.

LOCALISATION OF THE NEW *TaqI* RESTRICTION SITE

The sequence change producing the new *TaqI* site appears to be associated with an allele causing a deficiency of α -fucosidase. The new *TaqI* site results in the loss of a 5.0 kb band and the generation of a new 1.8 kb band. It was not possible to localise the new *TaqI* site by direct restriction mapping because the sizes of some of the intronic sequences are not known. However, it was possible to deduce which section of the gene corresponded to the 5 kb fragment by the use of probes that cover different regions of the cDNA sequence and knowledge of the sequence. The new *TaqI* site was not observed after hybridisation with probe A which covers exons 1, 2, and part of 3, indicating that it is not in this region of the gene. The 5.4 kb *TaqI* band is much less intense than other bands when probe B is used for hybridisation but of comparable intensity when probe A+B is used. The *HincII* site separating probes A and B is in exon 3, 5 bp upstream from the 3' end of exon 3 (fig 1). This suggests that the 5.4 kb fragment spans exon 3. The 5.4 kb fragment is unaffected in patient SB, thereby excluding exon 3 as the location of

the new *TaqI* site. Probe C, which covers the 3' end of exon 8 and the 3' non-coding region, only hybridises to the 2.4 kb fragment. As no *TaqI* sites are present in exon 7, intron 7, and exon 8, according to the published sequence the 2.4 kb fragment must span exons 7 and 8. As the 2.4 kb fragment is intact in patient SB, the new *TaqI* site cannot be in exons 7 and 8 or intron 7. The absence of the 2.4 kb *TaqI* fragment in patients with the 3' deletion that includes exons 7 and 8² supports this conclusion. Exon 4 can also be excluded for the following reason. Exon 4 is only 106 bp long and there is a *TaqI* site 158 bp upstream. As the new *TaqI* site has to be 1.8 or 3.2 kb from an existing *TaqI* site, it cannot be in or flanking exon 4. For these reasons the 5 kb *TaqI* fragment in normal DNA, which is split by the new *TaqI* site, must span exon 5 or 6. This was investigated by amplifying exons 5 and 6 and their flanking regions from the patient and controls by PCR (fig 2) and analysing the amplified molecules for sequence differences.

ANALYSIS OF EXON 6

Primers complementary to 5' and 3' intronic sequences flanking exon 6 (primers 1 and 2 in the table and fig 2) were used to prepare amplified DNA from SB and a control. Both amplification products failed to cut with *TaqI*, indicating that the new *TaqI* site in SB is not in exon 6. Further evidence that a new *TaqI* site or another sequence change was not present in exon 6 of SB was obtained by SSCP analysis of the amplified products from SB and controls (fig 5A). Confirmation of the identity of the sequences of exon 6 and its flanking regions in SB and controls was obtained by direct sequencing of the amplified products and comparison with the published sequence.

ANALYSIS OF EXON 5

DNA from the patient and controls was amplified using primers specific for the 5' and 3' ends of exon 5 (primers 3 and 4 in the table and fig 2). There are no *TaqI* sites in the 5' and 3' ends of exon 5 covered by the primers, according to the published sequence. The amplified material from both sources consistently failed to be cut by *TaqI*, suggesting that the new *TaqI* site was not in exon 5. SSCP analysis of amplified exon 5 from SB and controls also indicated that the sequence of exon 5 was the same in the patient and controls. This was confirmed by direct sequencing of the amplified products.

ANALYSIS OF INTRON 5

To amplify across the 5' splice site of intron 5, primers were synthesised specific for the middle of exon 5 and a sequence in intron 5 (primers 5 and 6 in the table and fig 2), which should result in a 269 bp product. The amplified product from controls did not cut with *TaqI*, whereas the product from SB was cut into two unequal fragments (fig 6). As the exonic portion of the PCR product was 201 bp

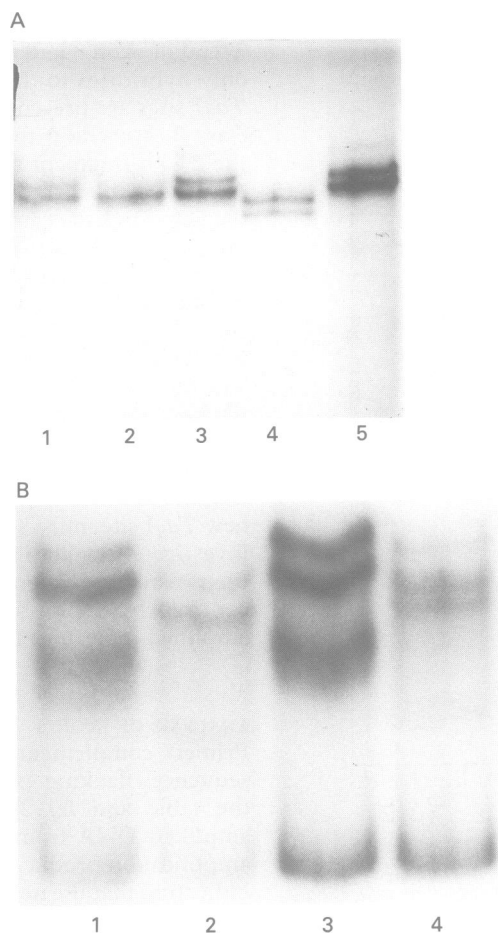


Figure 5 Single stranded conformational polymorphism analysis of amplified exons 5 and 6. (A) Exon 6. 1, 2, and 5, controls; 3, patient SB; 4, fucosidosis patient with a different exon 6 mutation (positive control). (B) Exon 5. 1 and 3, controls; 2, patient SB; 4, fucosidosis patient with a different exon 5 mutation (positive control). See Materials and methods and results for details.

in length and this was not cleaved by *TaqI*, the new *TaqI* site must be in the first 68 bases of intron 5. SSCP confirmed that there was a difference in sequences of the amplified products obtained with these primers from SB and controls (fig 5B). Sequencing showed a single base transition from G to A in SB at the first base in intron 5 (fig 7). This creates a new

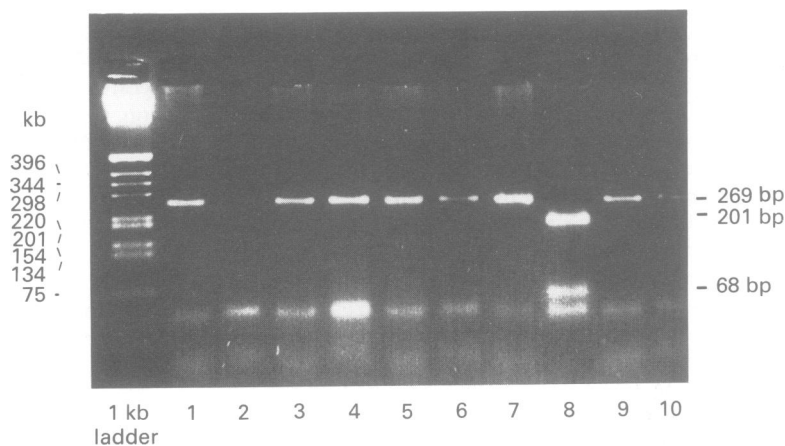


Figure 6 *TaqI* digestion of PCR amplified exon 5. 1 and 2 and 3 and 4: controls, respectively undigested and digested with *TaqI*; 5 and 6 and 9 and 10: other fucosidosis patients, undigested and digested; 7 and 8, patient SB, undigested and digested.

TaqI site of TCGA at the exon 5/intron 5 boundary and is consistent with the approximate sizes of the digestion fragments obtained with *TaqI* from the amplified material.

Discussion

Our patient with fucosidosis does not have either of the two previously described disease causing mutations in the α -fucosidase gene, the 3' deletion or the premature termination signal in exon 8. However, she is homozygous for a new *TaqI* polymorphism owing to a g \rightarrow a transition at position +1 of the exon 5/intron 5' splice site. This caused the loss of a 5.0 kb band and the appearance of a new 1.8 kb band. It is assumed that a 3.2 kb band is also generated but that it is undetectable on the blot, either because it comigrates with the 3.3 kb band, or more likely, because it is an intronic sequence and therefore not detectable with the cDNA probe. The absence of this sequence change in controls and its cosegregation with the enzyme deficiency in the family strongly suggest that it is the disease causing mutation in this child. The sequence g t is highly conserved at the 5' splice site of mammalian introns.¹⁷⁻¹⁹ A mutation of the g at the +1 position of a 5' splicing site consistently leads to abnormal processing of messenger RNA, either exon skipping or activation of a cryptic splice site.²⁰ 5' splice site mutations have been reported in genes for several other human lysosomal enzymes. These mutations lead to an almost complete deficiency of the enzyme when they occur homozygously in patients. A g \rightarrow a transition in the 5' splice site of intron 2 of the α chain of β -hexosaminidase has been found in a compound heterozygote with Tay-Sachs disease.²¹ Truncated mRNA lacking exon 2 is produced along with normal length mRNA from the normal allele. One of the two major mutations responsible for Tay-Sachs disease in Ashkenazi Jews is a g \rightarrow c transversion in the 5' splice site of intron 12 of the same gene, which leads to no detectable mRNA.²²⁻²⁴ In metachromatic leucodystrophy, g \rightarrow a transitions in the +1 5' splice site of introns 2²⁵ and 3²⁶ are present in mutant arylsulphatase A alleles associated with the more severe, early onset, late infantile form of the disease. A g \rightarrow t transversion in the +1 position of the 5' splice site of intron 6 of α -galactosidase resulted exclusively in skipping of exon 6 in a patient with Fabry disease.²⁰ A patient with Gaucher disease was also found to have a 5' splice site mutation in intron 2 of the β -glucocerebrosidase gene.²⁷

The possible consequence of a mutation in an intron 5' splice site can be assessed semi-quantitatively by comparing the statistical estimates for the probable use of the normal and mutant splice sites.¹⁹ Based on the -2 to +6 consensus sequence for a 5' splice site, the g \rightarrow a transition in the exon 5 donor splice site of α -fucosidase would cause a decrease in the likelihood score from 86 to 68. All the other 5' splice site mutations in lysosomal enzyme genes led to a very similar drop in the calculated likelihood score for the use of the splice site. This

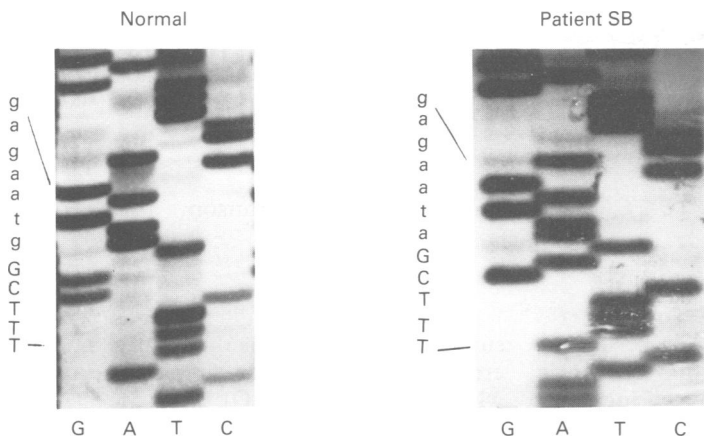


Figure 7 Sequencing of part of exon 5 and 5' flanking region, amplified by PCR showing the g→a substitution in SB.

strongly supports our suggestion that the mutation at the +1 position of the 5' splice site of intron 5 is the disease causing mutation in our patient. It would be expected to lead to incorrect processing of mRNA and a deficiency of functional enzyme. Unfortunately it has not been possible to study the mRNA or enzymic protein in this family.

To date only three disease causing mutations have been fully characterised in cases of fucosidosis. Therefore it is not yet possible to correlate the genotype with clinical phenotype. Like the patients with the 3' deletion or premature stop codon in exon 8, our patient has negligible α -fucosidase activity in her white blood cells and clinical symptoms were detected between 1 and 2 years of age. Historically fucosidosis was classified into type 1, which was characterised by a rapidly progressive neurological deterioration and death before the age of 5 years, and type 2, which had a slower progression. However, patients homozygous for the mutation in exon 8 have been classified as both type 1 and type 2 on clinical criteria.²⁸ Therefore it is possible that other factors contribute to the course of the disease.

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