Identification of 12 novel mutations in the α-N-acetylglucosaminidase gene in 14 patients with Sanfilippo syndrome type B (mucopolysaccharidosis type IIIB)

Clare E Beesley, Elisabeth P Young, Ashok Vellodi, Bryan G Winchester

Abstract
Sanfilippo syndrome type B or mucopolysaccharidosis type IIIB (MPS IIIB) is one of a group of lysosomal storage disorders that are characterised by the inability to breakdown heparan sulphate. In MPS IIIB, there is a deficiency in the enzyme α-N-acetylglucosaminidase (NAGLU) and early clinical symptoms include aggressive behaviour and hyperactivity followed by progressive mental retardation. The disease is autosomal recessive and the gene for NAGLU, which is situated on chromosome 17q21, is approximately 8.5 kb in length and contains six exons. Primers were designed to amplify the entire coding region and intron/exon boundaries of the NAGLU gene in 10 fragments. The PCR products were analysed for sequence changes using SSCP analysis and fluorescent DNA sequencing technology. Sixteen different putative mutations were detected in DNA from 14 MPS IIIB patients, 12 of which have not been found previously. The mutations include four deletions (219-237del19, 334-358del25, 1335delC, 2099delA), two insertions (1447-1448insT, 1932-1933insGCTAC), two nonsense mutations (R297X, R626X), and eight missense mutations (F48C, Y140C, R234C, W268R, P521L, R565W, L591P, E705K). In this study, the Y140C, R297X, and R626X mutations were all found in more than one patient and together accounted for 25% of mutant alleles.

Keywords: Sanfilippo syndrome type B; mucopolysaccharidosis IIIB; α-N-acetylglucosaminidase; mutations

Sanfilippo syndrome type B or mucopolysaccharidosis type IIIB (MPS IIIB) is a lysosomal storage disorder that is caused by the deficiency of the enzyme α-N-acetylglucosaminidase (NAGLU). NAGLU catalyses the removal of the N-acetylglucosamine residues from the non-reducing terminal of heparan sulphate during lysosomal degradation. The deficiency results in the accumulation inside lysosomes and excretion in the urine of heparan sulphate. Diagnosis is based on detection of excessive urinary heparan sulphate and by demonstration of a deficiency of NAGLU in white blood cells, plasma, or fibroblasts. Clinical symptoms are initially characterised by intractable hyperactivity and aggressive behaviour, followed by progressive mental retardation with death usually in the late teens. A more slowly progressive form of the disease with later onset, known as the attenuated phenotype, has been described in a small number of MPS IIIB patients.

MPS IIIB is autosomal recessive and the gene encoding the α-N-acetylglucosaminidase enzyme (NAGLU) has been characterised recently. The gene which is situated on chromosome 17q21, is approximately 8.5 kb in length and contains six exons. The cDNA is 8.2 kb long and encodes a protein of 743 amino acids. Characterisation of the genomic structure of the NAGLU gene (Genbank accession number U43572) has made it possible to start a mutational analysis. There is evidence of clinical variability among MPS IIIB patients and this variation may be the result, in part, of the involvement of different allelic mutations. There are 23 different mutations have been identified in the NAGLU gene from MPS IIIB patients including two deletions, two insertions, five nonsense mutations, and 14 missense mutations. There appears to be extensive heterogeneity in MPS IIIB. In this study, the NAGLU gene from 14 patients has been analysed by PCR, SSCP analysis, and automated DNA sequencing. Sixteen different mutations have been found, 12 of which were previously unknown. The novel mutations include four deletions (219-237del19, 334-358del25, 1335delC, 2099delA), two insertions (1447-1448insT, 1932-1933insGCTAC), and six missense mutations (F48C, Y140C, R234C, W268R, R565W, L591P, E705K).

Materials and methods
GENOMIC DNA EXTRACTION AND PCR
The group of 14 MPS IIIB patients were all diagnosed with the severe form of the disease, with a NAGLU enzyme activity in white blood cells of <0.1 nmol/h/mg protein (ref range 1.2-4.6). Age of onset was during early childhood. Genomic DNA was extracted from either venous blood or fibroblast cell lines of the patients using a modified version of the ammonium acetate salting out method.

Intronic primers were designed to amplify the coding region and intron/exon boundaries of the NAGLU gene. Exon 1 (715 bp) was amplified as two PCR products and exon 6 (1422 bp) as four products (table 1). The sense and antisense primers were tagged at their 5’ ends with the M13 (-21) forward primer
sequence (5'-TGTAAACACGACGGCACTG-3') and the M13 reverse primer sequence (5'-CAGGAAAAAGCTATGACACC-3'), respectively. These universal primer binding sites at the 5' and 3' termini of the PCR products were used for DNA sequencing.

A typical PCR reaction using 100 ng of genomic DNA contained 25 pmol of each primer, 1 x NH4 reaction buffer (Bioline), 4% (v/v) DMSO (dimethylsulphoxide), 0.2 mmol/l dNTPs, 0.5 μl (2.5 units) BioPro™ DNA polymerase (Bioline) (added after "hot start"). Details of annealing temperatures and MgCl2 concentrations for each particular amplification reaction are provided in table 1. Cycling conditions were typically 96°C for 10 minutes, followed by 35 cycles of one minute at 96°C, one minute at 60-64°C, one minute at 72°C, and a final extension at 72°C for 10 minutes.

SSCP ANALYSIS AND DNA SEQUENCING
Following amplification, the PCR products were subjected to SSCP (single strand conformation polymorphism) analysis using MDE™ gel (Mutation Detection Enhancement, FMC Bioproducts). The 10 PCR products were digested with a restriction enzyme before SSCP analysis (table 1). Two μl of a loading dye mixture (95% (v/v) formamide, 10 mmol/l NaOH, 0.25% (v/v) bromophenol blue, 0.25% (w/v) xylene cyanol) were added to 5 μl of digestion mix. The samples were denatured at 94°C for four minutes before loading onto a 0.5 x MDE™ gel. Electrophoresis was carried out in 0.5 x TBE at 15 W overnight at 4°C. Bands were detected using a silver staining procedure.12

Fragments of interest were concentrated and purified from excess primers and dNTPs by ultrafiltration through Microcon™-100 columns (Millipore) before sequencing. Products were sequenced in both the forward and reverse direction using the appropriate M13 dye labelled primer kits (Perkin Elmer Applied Biosystems). Reactions were performed as instructed and samples were analysed on an ABI Prism™ 377 DNA Sequencer (Perkin Elmer Applied Biosystems); 25% (v/v) DMSO was included in the sequencing reaction mixes for exons 1a, 1b, and 2 because of their high GC content.

Sequence changes were confirmed by either digestion with a restriction enzyme or by ACRS (amplification restriction site) PCR.13 Primer sequences, annealing

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Table 1 Primers and PCR conditions required for amplification of NAGLU gene

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Primer</th>
<th>Nucleotide position</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>SFB1a(+)</td>
<td>1292-1307</td>
<td>M13 (321) - GGAACCCGCGCCTCCTC</td>
</tr>
<tr>
<td>1b</td>
<td>SFB1b(+)</td>
<td>1451-1466</td>
<td>M13 (321) - TCCTTCCGCTCGG</td>
</tr>
<tr>
<td>2</td>
<td>SFB2(+)</td>
<td>2446-2450</td>
<td>M13 (321) - GCTGTCGGCCTCCTC</td>
</tr>
<tr>
<td>3</td>
<td>SFB3(+)</td>
<td>3388-3407</td>
<td>M13 (321) - CCAGACAAAGAAGACATG</td>
</tr>
<tr>
<td>4</td>
<td>SFB4(+)</td>
<td>3740-3758</td>
<td>M13 (321) - ATCCCTGGACAGTACCCG</td>
</tr>
<tr>
<td>5</td>
<td>SFB5(+)</td>
<td>4070-4091</td>
<td>M13 (321) - GGTGATGCGCCACC</td>
</tr>
<tr>
<td>6a</td>
<td>SFB6a(+)</td>
<td>8058-8074</td>
<td>M13 (321) - GGACAAAGCTAGCAGT</td>
</tr>
<tr>
<td>6b</td>
<td>SFB6b(+)</td>
<td>8495-8480</td>
<td>M13 (321) - CGCTGATGCTCCCTG</td>
</tr>
<tr>
<td>6c</td>
<td>SFB6c(+)</td>
<td>8402-8421</td>
<td>M13 (321) - CCTGAGGCTCTGAAAGAG</td>
</tr>
<tr>
<td>6d</td>
<td>SFB6d(+)</td>
<td>8800-8875</td>
<td>M13 (321) - GCCTGACGGAGGAG</td>
</tr>
</tbody>
</table>

*Positions of primers are numbered according to Genbank database entry U43572.a

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Table 2 Primers and PCR conditions for ACRS reactions

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Sense primer (nucleotide position)</th>
<th>Antisense primer (table 1)</th>
<th>Product size (bp)</th>
<th>Annealing temp (°C)</th>
<th>MgCl2 conc (mM)</th>
<th>Restriction enzyme (fragment sizes (bp))</th>
</tr>
</thead>
<tbody>
<tr>
<td>F48C</td>
<td>GGCTGTCGGACGCGCGCGGCGCA (1529-1558)</td>
<td>SFB1b(-)</td>
<td>439</td>
<td>64</td>
<td>1.5</td>
<td>+AluI (31+178+39+37+94+18+42)</td>
</tr>
<tr>
<td>219-237del19</td>
<td>CGGTGGCGGCGGGGCGCGGGCTCCACGGCA (1615-1663)</td>
<td>SFB1b(-)</td>
<td>334</td>
<td>68</td>
<td>1.5</td>
<td>+NlaIII (32+502)</td>
</tr>
<tr>
<td>W768R</td>
<td>CCTGACGCTTCAATGACAGATTGGCAGC (6098-6127)</td>
<td>SFB5(-)</td>
<td>316</td>
<td>64</td>
<td>1.5</td>
<td>+Nal (31+285)</td>
</tr>
<tr>
<td>R297X</td>
<td>ATATCCCGCCTGGGCGGCTCCTCCAG (6185-6214)</td>
<td>SFB5(-)</td>
<td>229</td>
<td>62</td>
<td>2.0</td>
<td>+BsrI (25+204)</td>
</tr>
<tr>
<td>1447-1448insT</td>
<td>CCTGGTGACAGCTTGGCAGCATGGGCAG (8563-8592)</td>
<td>SFB6(-)</td>
<td>257</td>
<td>64</td>
<td>1.5</td>
<td>-NalII (257)</td>
</tr>
<tr>
<td>R565W</td>
<td>GGGTGCCTGAGCTGCTGTCGGACCTACA (8808-8837)</td>
<td>SFB6(-)</td>
<td>392</td>
<td>62</td>
<td>2.0</td>
<td>+NalIII (28+364)</td>
</tr>
<tr>
<td>L591P</td>
<td>GGCGCTACCTGACGAGAAGGACTGCGTCCCG (8887-8916)</td>
<td>SFB6(-)</td>
<td>313</td>
<td>66</td>
<td>1.5</td>
<td>+BstUlI (30+283)</td>
</tr>
<tr>
<td>1932-1933insGCTAC</td>
<td>AGCCGTAGGTCTTGAAGCAGGAGAAGCGCGCGG (9046-9075)</td>
<td>SFB6(-)</td>
<td>159</td>
<td>64</td>
<td>1.0</td>
<td>+HpaI (39+120)</td>
</tr>
<tr>
<td>ET05K</td>
<td>CACCAATTTGACAAAAATGCTTCCACATT (9228-9257)</td>
<td>SFB6(-)</td>
<td>335</td>
<td>62</td>
<td>1.5</td>
<td>+AflIII (28+307)</td>
</tr>
</tbody>
</table>
temperatures, and MgCl₂ concentrations for the ACRS PCR reactions are provided in Table 2. All other parameters were as described for the typical PCR reaction.

### Results

The six exons and the intron/exon boundaries of the NAGLU gene from 14 patients with severe MPS IIIB were amplified by PCR. All of the 10 fragments were digested with a restriction enzyme before SSCP analysis. Fragments showing a shift were purified and sequenced directly using fluorescent DNA sequencing technology (Perkin Elmer Applied Biosystems). In patient samples where no only one heterozygous shift was observed (21.4%), all of the amplified PCR products were directly sequenced. Using these techniques, 16 different mutations were found, 11 of which have not been previously reported.

### Table 3 Mutations found in the NAGLU gene from patients with MPS IIIB in this study

<table>
<thead>
<tr>
<th>Exon (fragment)</th>
<th>Mutation*</th>
<th>Nucleotide alteration*</th>
<th>Protein alteration</th>
<th>SSCP shift</th>
<th>RE test</th>
<th>ACRS test (table 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (1a)</td>
<td>F48C</td>
<td>TAC→TGC</td>
<td>Phe→Cys</td>
<td>+</td>
<td>+Ahd</td>
<td></td>
</tr>
<tr>
<td>1 (1b)</td>
<td>219-237del19</td>
<td>19 bp del</td>
<td>41 altered aa, term +</td>
<td>+</td>
<td>+NlaII</td>
<td></td>
</tr>
<tr>
<td>1 (1b)</td>
<td>334-358del25</td>
<td>25 bp del</td>
<td>1 altered aa, term +</td>
<td>+</td>
<td>+NlaII</td>
<td></td>
</tr>
<tr>
<td>2 (2)</td>
<td>Y140C§</td>
<td>TAC→TGC</td>
<td>Tyr→Cys</td>
<td>+</td>
<td>+NlaII</td>
<td>+Fnu4HI</td>
</tr>
<tr>
<td>4 (4)</td>
<td>R234C</td>
<td>CGC→TGC</td>
<td>Arg→Cys</td>
<td>+</td>
<td>+NlaII</td>
<td></td>
</tr>
<tr>
<td>5 (5)</td>
<td>W268R</td>
<td>TGG→CGG</td>
<td>Trp→Arg</td>
<td>+</td>
<td>+NlaII</td>
<td></td>
</tr>
<tr>
<td>5 (5)</td>
<td>R297X†</td>
<td>CGA→TGA</td>
<td>Arg→Stop</td>
<td>−</td>
<td>+BsrI</td>
<td></td>
</tr>
<tr>
<td>6 (6)</td>
<td>1335delC</td>
<td>GC→TGC</td>
<td>29 altered aa, term +</td>
<td>+</td>
<td>+BglI</td>
<td>+NlaII</td>
</tr>
<tr>
<td>6 (6b)</td>
<td>1447-1448insT</td>
<td>1 bp ins</td>
<td>32 altered aa, term +</td>
<td>+</td>
<td>+NlaII</td>
<td></td>
</tr>
<tr>
<td>6 (6b)</td>
<td>P251L§</td>
<td>CGC→TGC</td>
<td>Pro→Leu</td>
<td>+</td>
<td>−EagI</td>
<td>+NlaII</td>
</tr>
<tr>
<td>6 (6c)</td>
<td>R565W</td>
<td>CGG→TGG</td>
<td>Arg→Trp</td>
<td>+</td>
<td>+NlaII</td>
<td></td>
</tr>
<tr>
<td>6 (6c)</td>
<td>L591P</td>
<td>CTG→CGG</td>
<td>Leu→Pro</td>
<td>−</td>
<td>+BstU</td>
<td></td>
</tr>
<tr>
<td>6 (6c)</td>
<td>R626X†</td>
<td>CGA→TGA</td>
<td>Arg→Stop</td>
<td>+</td>
<td>+SndI</td>
<td></td>
</tr>
<tr>
<td>6 (6c)</td>
<td>1932-1933insGCTAC</td>
<td>5 bp ins</td>
<td>3 altered aa, term +</td>
<td>+</td>
<td>+HglI</td>
<td></td>
</tr>
<tr>
<td>6 (6d)</td>
<td>2099delA</td>
<td>50 bp del</td>
<td>106 altered aa, term +</td>
<td>+</td>
<td>+AhdI</td>
<td>+AftI</td>
</tr>
<tr>
<td>6 (6d)</td>
<td>E705K</td>
<td>GAG→AAG</td>
<td>Glu→Lys</td>
<td>+</td>
<td>+AftI</td>
<td></td>
</tr>
</tbody>
</table>

*Number of codons and nucleotides according to ref 6.
†‡§Found previously by refs 6, 8, 9, respectively.

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**Figure 1** Six novel mutations in the genomic sequences of the NAGLU gene from patients with MPS IIIB. Sequence analysis was performed directly on PCR amplified genomic DNA using an ABI Prism™ M13 (-21) Dye Primer FS Cycle Sequencing Ready Reaction Kit, and products were run on an ABI Prism™ 377 DNA Sequencer (Perkin Elmer Applied Biosystems). (A) L591P; (B) W268R; (C) 2099delA; (D) 1447-1448insT; (E) 1335delC; (F) 334-358del25. Mutations A, C, and D are heterozygous, B, E, and F are homozygous. Base changes, insertions, and deletions are underlined. M=mutant sequence, N=normal sequence.
Among the 12 novel sequence changes there were four deletions and two insertions. In the larger insertions and deletions, analysis of the sequence adjacent to or within the affected region shows the presence of direct repeat elements which have probably played a role in the mutation event. In the 219-237del19 mutation, where there has been a deletion of 19 bp, there is a 7 bp direct repeat element (GCGGCGGC) just before the deletion site and at the 3' end of the deleted fragment. The 334-358del25 mutation, a deletion of 25 bp, shows a similar pattern with 5 bp direct repeat element TGCGG; however, there has been a deletion of an additional four G residues from the sequence just after the second direct repeat. There is a 3 bp direct repeat element GCT present in the vicinity of the 1932-1933insGCTAC mutation where five bases have been inserted between nucleotides 1932 and 1933. Two of the single base pair deletions, 2099delA and 1335delC, have occurred after a sequence of four A residues or four C residues, respectively. The 1447-1448insT mutation has occurred after a single T residue. All three have clearly arisen as a result of slipped mispairing during DNA replication. These insertions and deletions will be pathogenic since all of them cause a shift in the reading frame and five of the six will result in premature termination. The exception is 2099delA which occurs close to the C-terminus of the protein. The last 44 amino acids of the normal protein will be altered and an additional 22 amino acids added to the C-terminus (the new stop codon will be situated in the polyadenylation signal AT-TAAA). Since the C-terminus is a critical part of the protein, any alteration will affect its secondary structure and ultimately its function.

Two nonsense mutations were found in the group of patients studied, R297X and R626X, both of which have been previously reported. Only these two mutations and the pathogenic Y140C mutation were found in more than one MPS IIIB patient. Together they account for 25% of mutant alleles and are therefore relatively common. They occur in several different populations (table 4) and since the two premature stop mutations occur at CpG dinucleotides they have probably occurred independently in different parts of the world. In contrast, the Y140C mutation, which does not occur at a CpG dinucleotide, may be an ancient mutation. In the seven UK patients, the three “common” mutations account for 36% of mutant alleles and such information will be important for future screening of newly diagnosed MPS IIIB patients in the UK.

CpG dinucleotides in a gene sequence are known to be “mutational hotspots”. Of the 10 point mutations, five occur at a CpG dinucleotide. Eight of the point mutations were missense and none of the six novel missense mutations was found in 94 control chromosomes. In the patients who were homozygous for a particular missense mutation, no other sequence changes were found. This provides preliminary evidence for the pathogenic nature of these changes. Three of the mutations (W268R, R565W, E705K) alter the charge of

Table 3 lists these mutations and fig 1 shows the sequence data obtained for six of the mutations. R297X, R626X, Y140C, and P521L are mutations which have been reported previously. Of the 12 novel mutations, four are deletions (219-237del19, 334-358del25, 1335delC, 2099delA), two are insertions (1447-1448insT, 1932-1933insGCTAC), and six are missense mutations (F48C, R234C, W268R, R565W, L591P, E705K). Seven of the 16 sequence changes were confirmed by restriction enzyme digestion (table 3). For the remaining nine sequence changes, region specific primers were designed that would lead to the creation or loss of restriction sites at those known mutation sites, a technique known as ACRS PCR. Table 2 details the necessary amplification conditions for the ACRS reactions and the resulting restriction enzyme analysis.

Two putative mutations were identified in all 14 MPS IIIB patients. Table 4 details the genotype of the patients including their national origin. Eight of the patients were homozygous for their mutations and the remaining six were compound heterozygotes. Three of the mutations, Y140C, R297X, and R626X, were found in more than one patient.

The pathogenic nature of the six novel missense mutations on enzyme function has yet to be ascertained, but 94 control chromosomes were screened to determine if the changes were present in the general population. SSCP analysis was used to screen for four of the changes (F48C, W268R, R565W, E705K). Digestion with the restriction enzyme HhaI was used to detect R234C. ACRS PCR and BsrUI digestion was used to screen for L591P. None of the changes was found in any of the 94 control chromosomes. Two polymorphisms in which the amino acid sequence was not altered were found in the control chromosomes. They were R519R (AGG----AGA: 1557G>A) and P719P (CCG----CCA: 2157G>A). Each occurred in heterozygous form in a single control and both appear to be very rare (1.1%).

Discussion

Mutational analysis on 14 patients with the severe classical form of MPS IIIB has resulted in the identification of 16 putative mutations, 12 of which have not been previously described. Two putative mutations have been found in all 14 patients. Eight patients were homozygous for the sequence changes and the remaining six were compound heterozygotes.
highlight the family, pedigrees. Molecular
the number R674H) missense mutations
result in the substitution of residues
and shows NAGLU
future enzymes.
these presented
tions six novel
regions. Since
the transport
formation.
Two
mutations (F48C,
R297X, Y140C,
R626X, R674C,
R674H) have been found in more than
one family, the remaining 28 are, so far, confined
to individual pedigrees. These results further
highlight the heterogeneity of MPS IIIB at the
molecular level.

The authors would like to thank the Enzyme Laboratory of the Chemical Pathology Services at GOSH NHS Trust for carrying out the enzyme diagnosis of the MPS IIIIB patients. We thank Mr Alan Cooper and colleagues at the Willink Biochemical Genetics Centre in Manchester for supplying two of the patient samples. We are grateful for the help and support of the parents from The Society for Mucopolysaccharide Diseases in the UK. Financial support was provided by The Society for Mucopolysaccharide Diseases with funds raised by the charity Jeans For Genes. Funding for the ABI Prism™ 377 DNA sequencer was provided by The Wellcome Trust.

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