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Mucopolysaccharidosis IVA: Four New Exonic Mutations in Patients with N-Acetylgalactosamine-6-Sulfate Sulfatase Deficiency

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Summary

We report four new mutations in Japanese patients with mucopolysaccharidosis IVA (MPSIVA) who were heterozygous for a common double gene deletion. A nonsense mutation of CAG to TAG at codon 148 in exon 4 was identified, resulting in a change of Q to a stop codon and three missense mutations. V (GTC) to A (GCC) at codon 138 in exon 4, P (CCC) to S (TCC) at codon 151 in exon 5, and P (CCC) to L (CTC) at codon 151 in exon 5. Introduction of these mutations into the normal GALNS cDNA and transient expression in cultured fibroblasts resulted in a significant decrease in the enzyme activity. V138A and Q148X mutations result in changes of restriction site, which were analyzed by restriction-enzyme assay. P151S and P151L mutations that did not alter the restriction site were detected by direct sequencing or allele specific oligohybridization. Detection of the double gene deletion was initially done using Southern blots and was confirmed by PCR. Haplotypes were determined using seven polymorphisms to the GALNS locus in families with the double gene deletion. Haplotype analysis showed that the common double gene deletion occurred on a single haplotype, except for some variation in a VNTR-like polymorphism. This finding is consistent with a common founder for all individuals with this mutation.

Introduction

Mucopolysaccharidosis IVA (MPS IVA; classical Morquio disease) is inherited as an autosomal recessive disorder and is due to deficiency of the lysosomal enzyme N-acetylgalactosamine-6-sulfate sulfatase (GALNS; E.C.3.1.6.4) (Matalon et al. 1974; Singh et al. 1976; Di Ferrante et al. 1978; Horwitz and Dorfman 1978). Patients with MPS IVA may present with a broad spectrum of clinical phenotypes, from a severe form of progressive systemic bone involvement, hepatosplenomegaly, corneal opacities, short trunk dwarfism, coxa valga, odontoid hypoplasia, and a life span of only 20 or 30 years, to a mild form with normal life span, mild bone involvement, and mild visceral organ involvement (Morquio 1929; Maroteaux and Lamy 1967; Glössl et al. 1981; Orii et al. 1981; Fujimoto and Horwitz 1983; Hechit et al. 1984; Beck et al. 1986). Unlike most other forms of mucopolysaccharidoses, intelligence in MPS IVA is normal. Patients with the classical form of MPS IVA often die of cardiopulmonary complications or cervical cord compression. The GALNS enzyme is normally synthesized as a precursor glycopeptide of 120 kD. After cleavage of the signal peptide and carbohydrate modifications, mature enzyme subunits of 40 kD and 15 kD form the active, heterodimeric enzyme (Bielicki and Hopwood 1991; Masue et al. 1991). Studies of GALNS biosynthesis in fibroblasts from unrelated MPS IVA patients revealed several different types of defect, including no detectable synthesis of the enzyme precursor, synthesis of an unstable precursor protein and absence of the mature enzyme, and normal synthesis and processing of precursor to mature mutant protein (Masue et al. 1991). These findings suggested that a variety of mutations affect GALNS synthesis, maturation, and stability as well as subunit association and/or kinetic properties of the enzyme.

The full-length cDNA and genomic sequences encoding human GALNS have been isolated and characterized (Tomatsu et al. 1991; Nakashima et al. 1994). The gene spans ~35 kb in length, contains 14 exons and 13 introns, and was mapped to the region 16q24, by in situ hybridization (Tomatsu et al. 1992; Baker et al. 1993; Masuno et al. 1993). Multiple polymorphisms have been detected in the GALNS gene (Tomatsu et al. 1994*a*, 1994*b*, 1994*d*, 1995*c*, 1995*e*; Iwata et al. 1995). To

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date, several GALNS gene mutations responsible for various clinical phenotypes of MPS IVA have been identified, including missense, frame-shift, and splice-site mutations (Fukuda et al. 1992; Tomatsu et al. 1994*c*, 1995*a*, 1995*b*, 1995*d*; Ogawa et al. 1995). Large structural alterations have also been seen by Southern blot analysis (Tomatsu et al. 1994*d*, 1995*c*), and the breakpoints of one large rearrangement causing a double gene deletion have been determined in a Japanese homozygous patient (Hori et al. 1995).

In the present paper, we describe four novel mutations in the GALNS gene (one nonsense and three missense mutations) in four Japanese patients who are heterozygous for the common double deletion. Family analysis for each mutation was made using a PCR technique followed by restriction-enzyme digestion and Southern blot analysis. Haplotype analysis was used to search for the origin of this unique gene deletion.

Patients, Material, and Methods

Patients

Patient 1 was the product of a normal vaginal delivery, the second child of nonconsanguineous healthy parents. At age 3 mo, the patient presented with kyphosis and deformity of the breastbone. At age 6 years he had a waddling gate with a tendency to fall, corneal clouding, recurrent otitis media, and hepatomegaly. He was 106 cm tall, weighed 18 kg, and intelligence was normal. Enzyme assay revealed MPS IVA. At the age of 16 years, he can still walk with aid, goes to normal high school, and has substantial flexion-extension at the knees and hips. His phenotype is MPS IVA, intermediate form.

Patient 2 was born after a normal vaginal delivery, the first child of healthy nonconsanguineous parents with no history of skeletal disorders. He appeared normal at birth and up to the age of 2 years. At that time growth retardation and skeletal deformities were noticed, and he was diagnosed as MPS IVA, on the basis of clinical criteria, and this diagnosis was confirmed at age 3 years by biochemical analysis. At age 4 years, MPS IVA symptoms were more obvious, and at this time he was 91.6 cm tall and weighed 15.2 kg. He presented with hepatomegaly, corneal clouding, facial anomalies, conductive deafness, and some deformities of the skeleton (disproportional dwarfism, pectus carinatum, genu valga, kyphosis). At age 8 years he was treated by bone marrow transplantation, the donor being his younger brother. At the present age of 15 years, progressive skeletal anomalies have ceased and other extraskeletal manifestations have improved. The patient's phenotype was classical Morquio disease, severe form.

Patient 3 was the third child of healthy parents. There was no consanguinity, and no skeletal disorders had occurred in the family. The patient was delivered vaginally after an uncomplicated full-term pregnancy. In the newborn period and during the 1st year of life, psychomotor and mental development appeared normal. From ~18 mo of age, the parents noticed a progressive kyphosis, and at the age of 2 years his unusual gait together with a swelling of his hand and some deformities of his skeleton (disproportional dwarfism, pectus carinatum, genu valga) indicated MPS IVA. Confirmation was made by enzyme assay. At the age of 3 years, the patient was 80.4 cm tall and weighed 12.0 kg. He was intellectually normal but had decreased motor function and was unable to walk from the age of 10 years. He died of respiratory distress due to recurrent pneumonia at age 15 years. The patient's phenotype was MPS IVA, classical severe form.

Patient 4 was the first child of healthy parents. Neither consanguinity nor skeletal disorders could be proved. The vaginal delivery and the postnatal period were without any complication. When this child was 2 years old, the parents noticed strabismus and corneal clouding. At the age of 4 years, marked bone dysplasias (kyphosis, short trunk and neck, pectus carinatum, genu valgum, and disproportional dwarfism) led to the diagnosis of Morquio syndrome. Middle ear ventilation tubes were inserted three times from age 5 years to 18 years because of conductive deafness. From around the age of 10 years she had difficulty walking but had some flexion-extension ability at the knees and the hips, permitting her to walk with the aid of leg braces. At the age of 12 years she was unable to move, because of injury of the cervical cord. However, she goes to a normal high school, and her intelligence is preserved. The phenotype was classical Morquio disease, severe form.

Material

Restriction enzymes and DNA modifying enzymes were from Takara Shuzo, Toyobo, Boehringer Mannheim, Pharmacia, or BRL. [α-^{32}P] dCTP (specific activity, 3,000 Ci/mmol) was purchased from Amersham. Dideoxy cycle sequencing kits were from Applied Biosystems. PCR primers and specific oligonucleotide primers were synthesized using an automated DNA synthesizer (model 380A; Applied Biosystems). Hybond N membrane was from Amersham.

Cell Lines and Cell Culture

Primary culture of fibroblasts were established from skin biopsies, and MPS IVA fibroblast lines of four patients were from our diagnostic samples. Human fibroblasts were maintained in minimum essential medium supplemented with 10% fetal bovine serum, 1% penicillin, and 1 mg streptomycin/ml at 37°C. Peripheral blood

samples were obtained from the four family members with MPS IVA and from normal controls, with informed consent. Fibroblasts lines or peripheral blood samples of patients and their family members were used for Southern and northern blots and sequence analysis of mutations. A brief description is given below.

Southern and Northern Hybridization Analyses

Genomic DNA was isolated from fibroblasts or peripheral blood samples. Five micrograms of DNA samples were digested with *Bam*HI, *Eco*RI, *Hin*dIII, *Sac*I, and *Pst*I to completion, under conditions recommended by the supplier, electrophoresed in 0.8% agarose, transferred onto nylon membrane, and analyzed using the full-length GALNS cDNA or eight genomic subfragments covering the entire coding region of the GALNS gene as a probe. To determine the relative sizes and amounts of the 2.3-kb GALNS transcript, total RNA was isolated from fibroblasts, using the guanidium isothiocyanate procedure (Chomczynski and Sacchi 1987). RNA samples were electrophoresed in 1.1% agarose, by using the glyoxal method, transferred onto nylon membrane filters, and hybridized with full-length GALNS cDNA.

PCR Amplification, Screening by SSCP, and DNA Sequencing

Localization of mutations was obtained by screening 14 amplified genomic fragments for each exon by single-strand conformation polymorphism (SSCP), as described elsewhere (Nakashima et al. 1994). Here we studied only exons 4 and 5 because all other exons were normal by SSCP analysis. PCR was carried out using the forward primer OMF 117 (5′-TCTTGGGAAGTGCCATGCCCTGT-3′) and the reverse primer OMF 118 (5′-CTGGGCAGGCGTGGCCAGGAGACTT-3′) for exon 4 and the forward primer OMF 119 (5′-TTGCTGCTCAGAACTTCCGAGTGTC-3′) and the reverse primer OMF 120 (5′-CCAGGCAGGAGCCACATAGGCTCG-3′) for exon 5. SSCP analysis of PCR products was performed according to Orita et al. (1989), with minor modifications. The PCR mixture in 10 μl of total volume contained 1 pmol of each unlabeled primer, 12.5 μM each of the four deoxynucleotide triphosphates, 50 ng of genomic DNA, and 0.25 U of *Taq* DNA polymerase and 0.1 μCi [α-^{32}P] dCTP (Amersham). The PCR conditions for exons 4 and 5 were 2 min at 94°C (denaturing), followed by 30 cycles of 1 min at 94°C and 1 min at 72°C. A portion of the reaction mixture (1 μl) was used and mixed with 9 μl of SSCP loading buffer (95% formamide, 10 mM NaOH, 0.05% bromophenol blue, 0.05% xylene cyanol), and heated at 95°C for 2 min; 2 μl of sample were loaded onto an MDE gel (mutation detection enhancement gel; AT Biochem). Electrophoresis was performed at 30 w for 12 h at room temperature. The gel was dried onto filter paper and exposed to x-ray film at −80°C for 16 h. Following SSCP screening, the positive products were remade by PCR. The amplification mixture contained 1 μg of genomic DNA, 50 mM KCl, 10 mM Tris · HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 μM concentration of dNTPs, and 50 pmol of each sense and antisense primer in a reaction volume of 100 μl. Each reaction mixture was incubated at 94°C for 2 min, as a pretreatment, after the addition of 1–2.5 U of *Taq* polymerase. Forty cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min were carried out. Then PCR fragments were isolated from agarose gels, were subcloned into T-vector (Stratagene), and were sequenced in both directions by the fluorescent dideoxy cycle sequencing. For each product, at least 8–10 clones were sequenced.

Construction of Mutant GALNS Expression Plasmid and Transient Transfection into Deficient Fibroblasts

The wild-type GALNS cDNA (Tomatsu et al. 1991) was subcloned into pUC 13, and in vitro mutagenesis was performed with mutant allele-specific oligonucleotides (ASOs) corresponding to mutations V138A, Q148X, and P151S according to the supplier's recommendations (Clontech). The mutations were introduced into the GALNS expression vector by subcloning DNA restriction fragments from the pUC 13 clones carrying the respective mutation. DNA sequences of the resulting mutant GALNS-expression plasmids of pMF V138A, pMF Q148X, and pMF P151S were confirmed for the entire subcloned mutant fragments. Mutant GALNS-expression plasmids were transfected into GALNS-deficient fibroblasts by the liposome mediated transfection method (Gene Transfer; Tomatsu et al. 1991), and 120 h later, the transfected fibroblasts were collected and assayed for GALNS activity.

GALNS Enzyme Assays

GALNS activity was determined in cultured fibroblasts obtained from MPS IVA patients and transfected fibroblasts, using the substrate trisaccharide 6-sulfo-N-acetylgalactosamine-glucuronic acid-6-sulfo-N-acetyl-[1-^{3}H] galactosaminitol as described elsewhere (Glössl and Kresse 1978; Sukegawa and Orii 1982). The standard reaction mixture contained 1.0 nmol substrate (~60,000 dpm), 10 μl 120 mM sodium acetate buffer (pH 3.8), 5–20 μg protein of cell extract and 10 μg bovine serum albumin in a final volume of 60 μl. After incubation for 2 h at 37°C the reaction was stopped by the addition of 500 μl ice-cold water, and the preparation was loaded onto a 0.6-ml column of Dowex 1 × 2, 200–400 mesh, Cl^- form prepared in Pasteur pi-

pets. The column was washed with 5.0 ml of 0.4 M NaCl and the monosulphated trisaccharide product and was collected in a scintillation vial, and the remaining substrate was eluted by putting 5.0 ml of 0.9 M NaCl into a second scintillation vial. The sum of the radioactivity in the two vials was used to calculate the enzyme activity.

Haplotyping

GALNS gene haplotypes for each family were constructed through analysis of 7 RFLPs (fig. 1). Haplotypes for each subject were constructed by segregation analysis, on the basis of the assumption that there were no recombinational events at the GALNS locus within a given family.

The first polymorphism consists of a *Sty*I RFLP (CCA/TA/TGG) within the fifth intron (IVS 5 + 134; CAA [allele A] or CGA [allele a]) (Tomatsu et al. 1995*e*). The second polymorphism is located within the exon 7 (764 from A of the ATG initial codon on the cDNA). This polymorphism (ACG [allele B] or ATG [allele b]) affects a *Sph*I restriction endonuclease site (GCATGC) (Tomatsu et al. 1994*a*). The third polymorphism of a *Rsa*I restriction enzyme site (GTAC) is positioned within the seventh intron (IVS7 nt-90; GTAC [allele H] or GAAC [allele h]) (Tomatsu et al. 1995*a*). The fourth polymorphism is located at 1487 from A of the ATG initial codon within exon 13. This polymorphism (AAG [allele D] or AGG [allele d]) changes a *Stu*I restriction endonuclease site (AGGCCT) (Tomatsu et al. 1995*e*). The fifth polymorphism (CAG [allele E] or CGG [allele e]) consists of a *Hap*II RFLP (CCGG) found in exon 14 (Tomatsu et al. 1994*b*). These five RFLPs products of PCR amplification of each exon and its boundary were digested with their respective restriction enzymes and subsequently were separated on a 8.0% acrylamide gel. The sixth polymorphism consists of a *Xho*I RFLP downstream of the last exon 14 and reveals either a 14.0-kb (allele F1) or 4.0-kb (allele F2) fragment (Tomatsu et al. 1994*a*). The seventh polymorphism results from VNTR-like elements ranging from upstream of exon 9 to downstream of exon 9. At least five alleles were detected by a *Bam*HI restriction enzyme (Tomatsu et al. 1994*d*; Tomatsu et al. 1995*c*; Iwata et al. 1995). The last two RFLPs were identified by Southern blot analysis, using a respective genomic fragment as a probe.

Molecular Characterization of GALNS Mutations in Families

Genomic DNAs from family members were used to study transmission of mutations. The V138A sequence change within exon 4 in patient 1 was identified by PCR and restriction endonuclease cutting. This T-to-C transition introduces a new *Nae*I endonuclease restriction site: GTCGGC→GCCGGC. Primer sets and PCR conditions for amplification of exon 4 were described above. The PCR product is a 299-bp fragment. Restriction digests were analyzed on an 8% acrylamide gel and electrophoresed in 1 × TBE buffer for 3 h at 180 V. After electrophoresis, the DNA bands were visualized by staining with ethidium bromide (fig. 2). The Q148X nonsense mutation within exon 5 was detected using the described primer set and conditions for amplification by PCR. The C-to-T transition in exon 5 at codon 148 produces a new *Mae*I cut site (CCAG→CTAG), which was used to determine molecular genotype in this family.

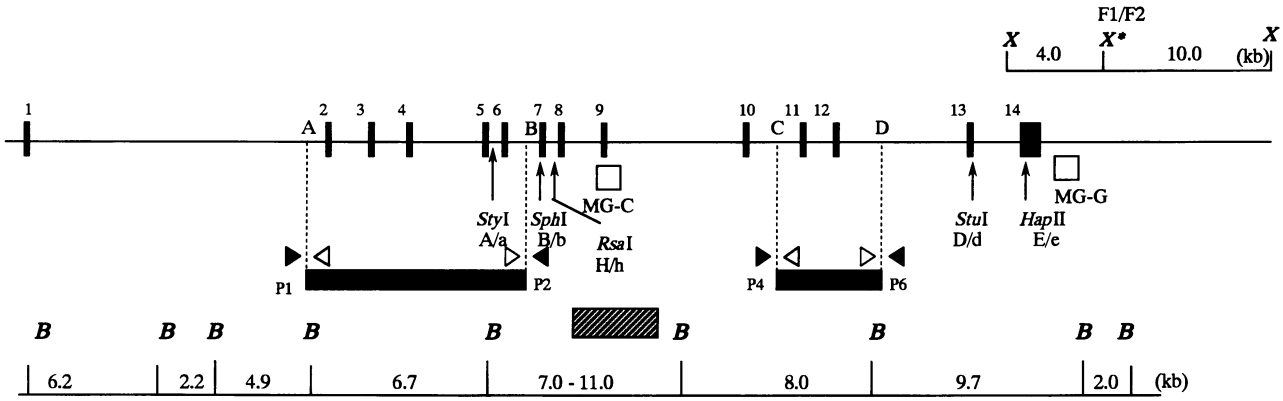


Figure 1 *Bam*HI restriction mapping of the GALNS gene with 14 exons and positions of genomic probes used for Southern blots. The italic *B* and *X* indicate *Bam*HI and *Xho*I sites, respectively. Unblackened boxes show the positions of genomic probes (MG-C and MG-G). An asterisk (*) shows a polymorphic site of *Xho*I, and the striped box, the possible location of a VNTR-like polymorphism. A blackened bar indicates deleted regions; the horizontal blackened arrowheads, the positions of primers for detection of the breakpoints of deletions 1 and 2; and the vertical arrows, the polymorphic sites. The horizontal unblackened arrowheads show the additional primers used for determination of the sequences around the breakpoints in normal controls. A, B, C, and D represent breakpoints.

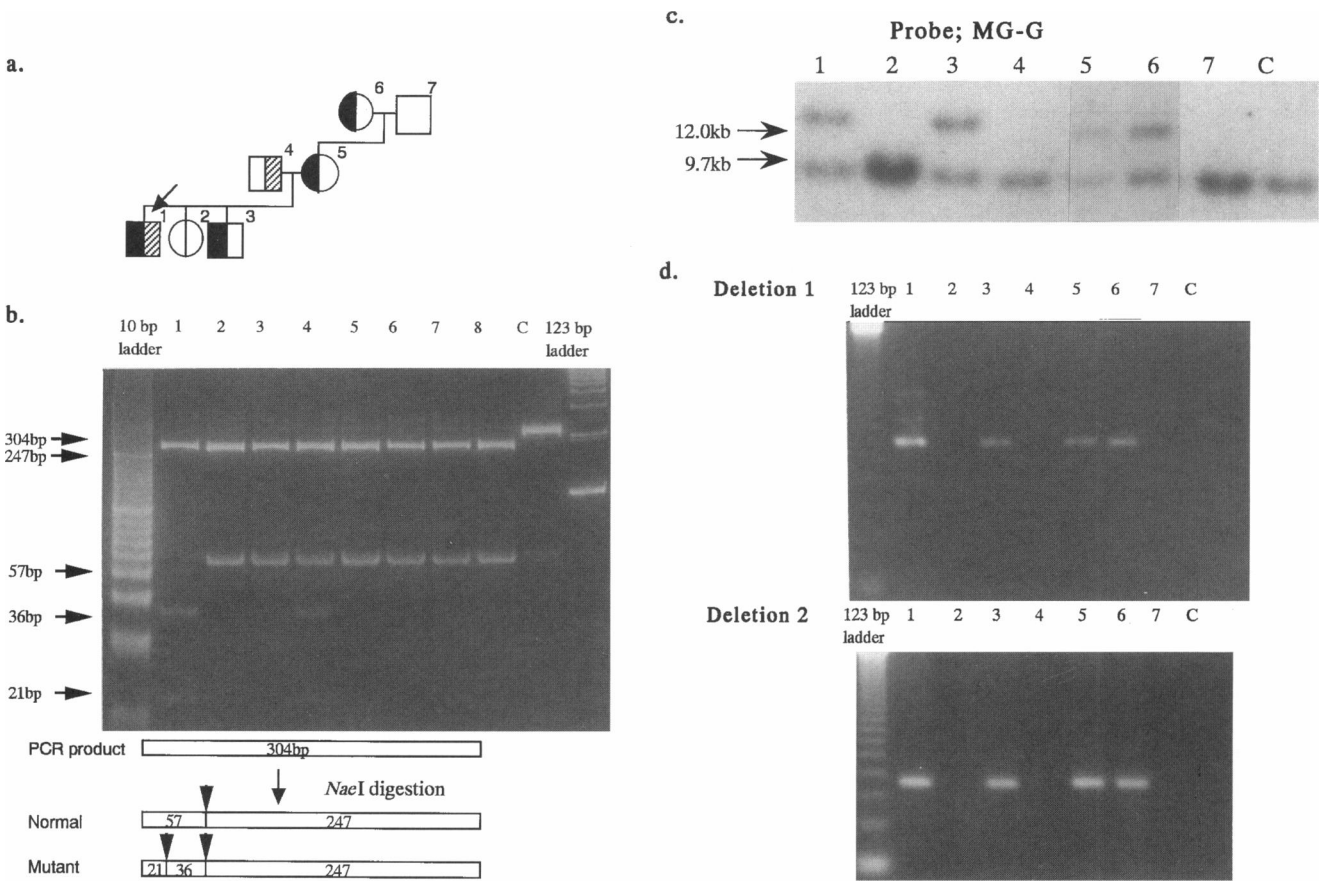


Figure 2 Analysis of family analysis of patient 1. *a,* Pedigree shows segregation of double-deletion allele (blackened symbols), V138A allele (hatched symbols), and wild-type allele (unblackened symbols). Arrow designates proband. *b,* Screening for the V138A allele in family members by restriction-enzyme analysis. The diagram below the photograph of the PAGE gel shows the fragment sizes expected following *Nae*I digestion of the amplified fragments containing exon 4 from the normal and V138A mutant alleles. The ethidium bromide–stained gel shows the results of analyses of the seven members of the pedigree shown in *A* (1–7), and unrelated normal (8), and the undigested 304-bp PCR fragment (C). *c,* Screening for the double-gene-deletion allele by restriction-enzyme analysis and Southern blotting. Southern hybridization analysis of *Bam*HI-digested total genomic DNA from pedigree members 1–7 and an unrelated control (C) revealed the normal 9.7-kb band in all samples, and an additional 12-kb band in members with a double-deletion allele (1, 3, 5, and 6) when hybridized with probe MG-G (the normal 7.5-kb and the unique 12.0-kb fragments were hybridized with probe MG-C in the same family members; data not shown). *d,* Detection of fragments spanning the breakpoints of deletions 1 and 2 by PCR amplification using primers P1 and P2 and P4 and P6, respectively. Only DNA from pedigree members with the double-deletion allele have the 836-bp PCR fragment spanning the breakpoint of deletion 1 and a 437-bp fragment spanning deletion 2.

The digested fragments were applied to an 8% polyacrylamide gel and electrophoresed in 1 × TBE buffer for 3 h at 180 V, followed by staining with ethidium bromide (fig. 3).

The P151S and P151L missense mutations at the same codon within exon 5 did not alter any restriction endonuclease site, and direct sequencing and ASO hybridization were used to confirm the mutation in the family tree (figs. 4 and 5). PCR-amplified DNA (411 bp) containing exon 5 was produced using the primers listed above and purified using a Centricon (Amicon) according the manufacturer's directions. Purified amplified DNA fragments were subsequently sequenced directly, without subcloning, by fluorescent dye terminator cycle sequencing (Applied Biosystems). Amplification primers were also used as the sequencing primers. ASO analysis for P151L and P151L mutations was performed using the same PCR-amplified fragments of genomic DNA from patients, and 1–2 μl of the product were denatured in 100 μl 10 mM Tris-HCl, pH 7.5, containing 0.2 M NaOH, for 10 min at room temperature. This mixture was neutralized with 100 μl 2 M NH_4OAc and was transferred onto Hybond N nylon membrane (Amersham), using a dot-blot apparatus (Bio-Rad Laboratories). The resultant membrane was fixed by the illuminator for 5 min, was prehybridized at 37°C for 3 h in 10-ml buffer made from hybridization buffer tablet (Amersham), and then was hybridized overnight at

37°C, in the same buffer containing [γ-^{32}P] ATP-labeled oligonucleotides.

Oligonucleotides used were as follows: normal oligonucleotide for P151S mutation (5′-CCAGTTCCACCCCCTGAAGCA-3′) and the mutant P151S oligonucleotide (3′-GGTCAAGGTGAGGGACTTCGT-5′), normal oligonucleotide for P151L mutation (5′-CAGTTCCACCCCCTGAAGCAC-3′) and the mutant P151L oligonucleotide (3′-GTCAAGGTGGAGGACTTCGTG-5′). After hybridization, the membrane was washed in 2 × SSC containing 0.1% SDS at 37°C for 15 min twice, followed by washing in 2 × SSC containing 0.1% SDS for 30 min twice at 58°C, which distinguishes between perfect matches and single-base mismatches. The membrane was washed, dried, and autoradiographed for 2 h at room temperature.

We used PCR to detect double gene deletion. DNA sequences flanking the breakpoints of double gene deletions in four families were amplified by PCR technique, using genomic DNA. Detailed sequences of each breakpoint for the gene deletions will be reported elsewhere. Sense and antisense primers for each deletion were prepared, and an 837-bp region and a 439-bp region containing the deletion 1 and deletion 2, respectively, were amplified. The sense primer for deletion 1 (P1; 5′-ACCTGGCACAAGTGGTTGGTCACT-3′) corresponded to 2 nt within intron 1. The antisense primer for deletion 1 (P2; 5′-ATACTGCTGGTTGAGAACTGGCGT-3′) was complementary to 24 nt within intron 5. Amplification for deletion 2 region containing the recombination junction was accomplished using a sense primer (P4; 5′-CTCAGCCTCCCAAGTAACTGGTAC-3′) constructed to 24 nt within intron 10 and an antisense primer (P6; 5′-GCCGTGCCTGTCTCTTAATGAAGT-3′) complementary to the 24 nt within intron 12. The buffers and concentrations of the reagents for PCR were identical to those used for exon amplification described above. Conditions for amplification were initial denaturing at 94°C for 2 min, 40 cycles of denaturation at 93°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The PCR products were subcloned into T-vector and sequenced to verify the authenticity.

Results

Identification of Common Double Gene Deletion in Four MPS IVA Patients

Four unrelated patients, three severe and one intermediate in phenotype, were studied. Northern blot analysis showed only a single band of apparently normal size message of 2.3 kb, in all patients. The intensities of GALNS mRNA bands were normalized by calculating their ratios to the intensities of actin mRNA bands, and the amount of the GALNS mRNA was only half normal in all MPSIVA fibroblasts used in this study. Figure 1 shows the structural organization of the GALNS gene and the *Bam*HI restriction fragments detectable by Southern blot analysis. The relative positions of two independent gene deletions and the polymorphic sites are also seen. Southern hybridization analysis in four patients with eight genomic subfragments identified the same rearrangement with two independent gene deletions. This common rearrangement was detectable with three additional restriction enzymes (e.g., *Xho*I, *Eco*RI, and *Hin*dIII). For further characterization of this rearrangement, Southern blot analysis of family members was done (the results of two representative families are shown in figs. 2 and 3). A *Bam*HI restriction enzyme was shown to clearly distinguish the mutant allele for molecular pedigree analysis (figs. 2 and 3). When probes MG-C and MG-G depicted in figure 1 were used, all four patients were seen to have normal restriction fragments and also a novel fragment, as compared with controls. Thus, these patients are heterozygous for this common rearrangement. In patients and heterozygous family members, hybridization with probe MG-G showed abnormal 12.0-kb and normal 9.7-kb *Bam*HI fragments (figs. 2*b* and 3*b*) and hybridization with probe MG-C, abnormal 11.5-kb to 12.3-kb *Bam*HI fragments in addition to normal 8.0-kb to 11.3-kb fragments (results are not shown in patients 3 and 4). The variation of fragment sizes is due to VNTR elements.

In the previous study, the deletion breakpoints were determined (see fig. 1); deletion 1 had a 5′ breakpoint in intron 1, ~2.5 kb upstream of exon 2 (A), and a 3′ breakpoint in intron 5, ~300 bp upstream of exon 6 (B), resulting in an 8.0-kb deletion. Deletion 2 had the 5′ breakpoint in intron 10, ~900 bp upstream of exon 11 (C), while the 3′ breakpoint was positioned in intron 12, ~1.5 kb upstream of exon 13 (D), eliminating nearly 6.0 kb of the genomic region. The deletion breakpoints were confirmed using PCR amplification. Figure 1 shows the positions of primers used to amplify the genomic fragment spanning the deletion breakpoints. The region around each breakpoint A, B, C, and D in both normal Japanese and Caucasians was also PCR-amplified and sequenced. There was no nucleotide change in any breakpoint region in a either population.

Identification of Exonic Point Mutations in GALNS Gene of Four MPS IVA Patients

To identify the other mutant alleles in these patients, we extracted genomic DNA and PCR amplified each of the 14 exons. We screened all exons and splice junctions of the patients' GALNS genes by SSCP and compared findings with those from normal controls. We found an abnormal pattern only in exon 4 for patient 1 and exon

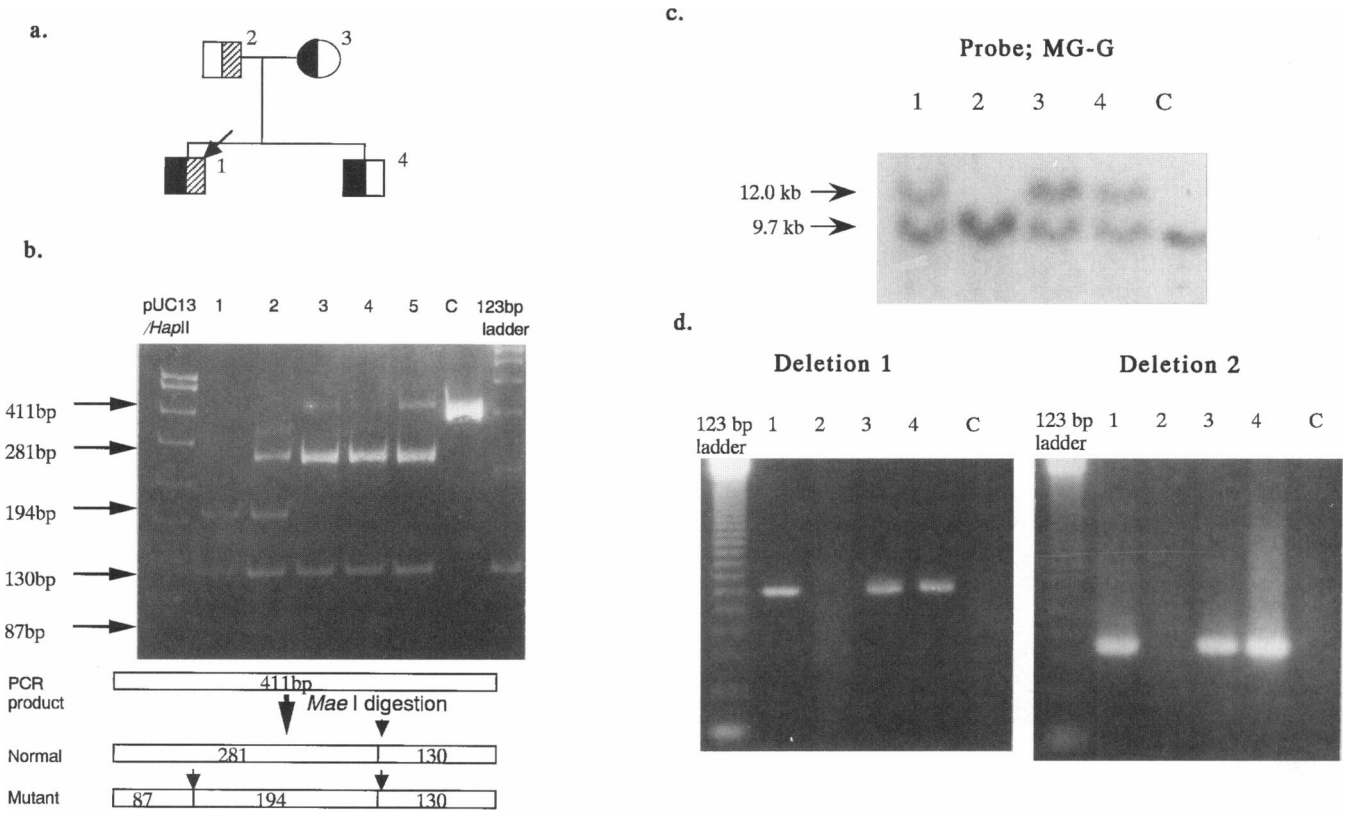


Figure 3 Analysis of family analysis of patient 2. *a*, Pedigree showing segregation of the double-deletion allele (blackened symbols), Q148X allele (hatched symbols), and normal allele (unblackened symbols). *b*, Screening for the Q148X allele by restriction-enzyme analysis. The diagram below the photograph of the ethidium bromide–stained gel shows the expected sizes of fragments produced by *Mae*I digestion of the 411-bp PCR-amplified fragments containing exon 5 from normal and mutant alleles. The 8% agarose gel separates the fragments obtained from *Mae*I-digested DNAs from pedigree members 1–7, an unrelated normal (8), and an undigested 411-bp fragment. *c*, Screening for the double-gene-deletion allele by Southern blot analysis of *Bam*HI-digested DNA using probe MG-G. The normal 9.7-kb band is seen in all DNA samples, and the 12-kb band is seen in DNA from pedigree members carrying the double-deletion allele (1, 3, and 4) (members 1, 3, and 4 were also found to contain the expected 11.8-kb band characteristic of the double-deletion allele with probe MG-C; data not shown). *d*, Detection of fragments spanning deletions 1 and 2 in DNA of individuals carrying the double-gene-deletion allele. Primers P1 and P2 produced 837-bp fragments, and P4 and P6 a 437-bp fragment spanning deletions 1 and 2, respectively.

5 for patients 2, 3, and 4 (fig. 4, *left*). After removal of the amplification primers, each DNA fragment was subcloned into T-vector, and the sequence of the respective exon and the splice junction was determined using an automated, nonradioactive sequencing method (for details, see Patients, Material, and Methods). In patient 1, the sequence of exon 4 revealed a single base substitution, a T-to-C transition at nt 469, which predicted a valine-to-alanine substitution (V138A; fig. 4, *bottom*). In patient 2, a C-to-T transition created a nonsense mutation that changed CAG (Gln) to TAG (Stop) at codon 148 in exon 5 (fig. 4, bottom). In patients 3 and 4, two different nucleotide exchanges were detected at the CCC codon, encoding proline 151. Different C-to-T transitions at nt 507 (patient 3) and nt 508 (patient 4) resulted in different amino acid substitutions of serine (TCC) and leucine (CTC), respectively (fig. 4, *bottom*). All five subclones sequenced in each patient contained a mutant allele and seemed to be homoallelic for each mutation, since exons 4 and 5 on the other alleles were deleted.

Confirmation of V138A, Q148X, P151S, P151L Mutations

To confirm the authenticities of the V138A, Q148X, P151S, and P151L mutations, restriction-enzyme analysis or dot-blot hybridization analyses were performed on PCR-amplified genomic DNA from patients 1, 2, 3, and 4 and from their family members. The V138A mutation creates an *Nae*I restriction endonuclease site (GTCGGC→GCCGCC). Normally, a constitutive *Nae*I restriction site causes the original 304-bp fragment to be cleaved to 247-bp and 57-bp fragments. A new *Nae*I site is created in the 57-bp fragment when the T-to-C transition at nt 469 is present, resulting in 36-bp and

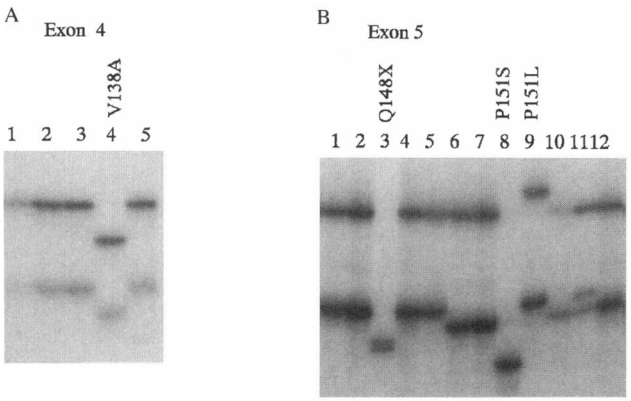
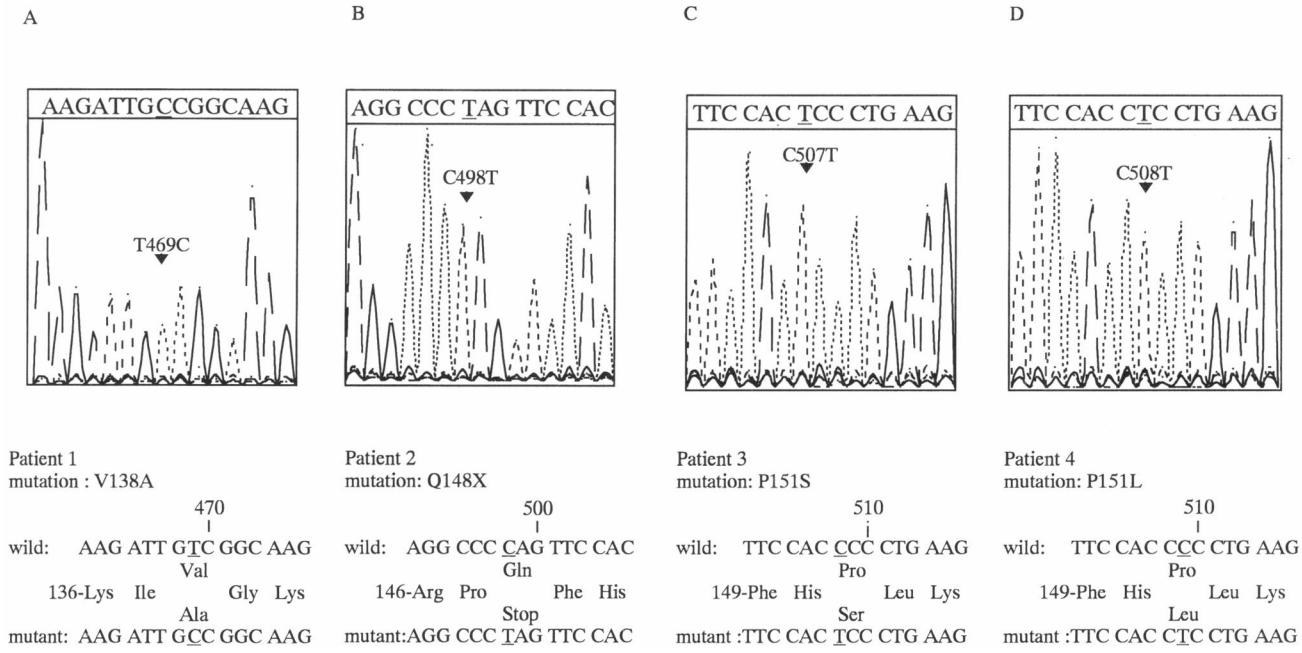


Figure 4 *Left,* SSCP analysis of exons 4 and 5 of the GALNS gene. Samples were amplified by PCR and run on an MDE gel at 30 W for 12 h at room temperature. *A,* the SSCP pattern of exon 4 for the V138A mutation is indicated in lane 4. Lanes 1–4 provide the MPS IVA samples, and lane 5 is for the control individual. *B,* the SSCP pattern for the present mutations in exon 5 are shown (lane 3, Q148X; lane 8, P151S; and lane 9, P151L). Lanes 1–10 indicate SSCP pattern from MPS IVA patients. The pattern of exon 5 is polymorphic because of *Sty*I polymorphism (A/a; shown by horizontal arrows), even in control individuals (lanes 11 and 12). The SSCP conditions are described in the text. *Below,* Identification of point mutations in GALNS-gene exons of four MPS IVA patients. Each amplified DNA fragment was subcloned and sequenced on both strands by fluorescent dye primer cycle sequencing and analysis on an automated DNA sequencer (see Patients, Material, and Methods). Sequencing diagrams covering positions of each nucleotide alteration are shown. The nucleotide sequences are given above the graphs. Only the mutant sequence is shown; the position of each mutation is marked by an arrowhead (panels A–D). In the lower half of each panel, wild-type and mutant nucleotide and amino acid sequences are given (amino acid numbering is on the left, and nucleotide numbering is above the normal sequences, both according to Tomatsu et al. [1991]).



21-bp fragments in place of the 57-bp fragment. *Nae*I endonuclease cutting of amplified exon 4 suggested that the proband was homozygous for this mutation, and the father heterozygous (fig. 2*b*). Genotyping for the Q148X codon change in exon 5 in the family pedigree of patient 2 was accomplished as outlined in figure 3*b*. The C-to-T transition creates a new *Mae*I restriction-enzyme site (CCAG→CTAG). In the presence of this missense mutation, the 411-bp PCR fragment was cleaved by *Mae*I into three fragments of 194, 130, and 87 bp, while only two fragments of 281 bp and 130 bp are produced in the absence of the Q148X. The results of *Mae*I digestion revealed that the proband was apparently homoallelic and that the father was heteroallelic for this mutation. For independent confirmation of the detected P151S (CCC→TCC) and P151L (CCC→CTC) mutations that did not alter any restriction site, the PCR DNA fragments of exon 5 in family members of patients 3 and 4 were dot blotted and hybridized to normal and P151S- or P151L-specific radiolabeled oligonucleotides. The PCR-amplified genomic DNA from patient 3 hybridized to the mutation-specific oligonucleotide but not to the normal ASO, confirming the authenticity of the C-to-T transition and indicating that patient 3 was apparently

homoallelic for the P151L mutation. Of the five other family members studied, only the father was heterozygous for this mutation. Dot-blot hybridization for the family of patient 4 confirmed the P151L mutation of patient 4 in an apparently homoallelic state and demonstrated that the P151L mutation was transmitted from the father (results are not shown).

Occurrence of the Common Double Gene Deletion in MPS IVA and Haplotype Analysis

The occurrence of the common double gene deletion in MPS IVA patients of Japanese and Caucasian origin was determined by Southern blot and PCR analyses. This mutation was observed in six (15.0%) of alleles of Japanese MPS IVA patients. However, of >100 GALNS alleles from Caucasian MPS IVA patients, none had the double-gene-deletion mutation. Haplotypes were determined for all five patients reported here, by using the seven polymorphic sites of the GALNS gene on DNA from pedigree members (fig. 1). A *Sty*I RFLP in the fifth intron was not useful, because this polymorphic region was deleted. No single recombination event was detected within the gene. If we eliminate the effect of VNTR-like polymorphisms on haplotype analysis, five other RFLPs were identical, showing that the common haplotype was b/D/E/F1/h. Although this haplotype was also a frequent one among normal control alleles (6 [20%]/32 chromosomes; table 1), analysis of the haplotype frequency in the normal population and in MPS IVA patients revealed the presence of linkage disequilibrium ($\chi^2 = 5.170$, df $= 1$, $P < .05$).

Characterization of Transiently Expressed GALNS Activity of Mutants

To investigate the effect of amino acids substitutions or termination on enzyme activity of the encoded GALNS polypeptides, the respective nucleotides were introduced into the wild-type GALNS-expression plasmid pMFN by site-directed mutagenesis. The resulting mutant cDNA expression plasmids pMF V138A, pMF Q148X, pMF P151S, and pMF P151L were transfected into GALNS-deficient fibroblasts. The fibroblasts transfected with mutant cDNAs showed almost complete loss in the enzyme activities (0.2–0.7 ± 0.2 nmol/mg protein/h), as compared with fibroblasts with normal cDNA (20.2 ± 3.0 nmol/mg protein/h). pMF V138A, which was expected to produce a milder phenotype, also showed a complete loss in the activity (0.2 ± 0.1 nmol/mg protein/h). There was no significant difference in the GALNS expression between three other mutant (0.5–0.7 ± 0.3 nmol/mg protein/h) cDNAs leading to a severe phenotype.

Table 1

Haplotype Frequencies of Normal, Mutant, and Double-Gene-Deleted Alleles

Allele	Normal Alleles (*n*)		MPS IVA Alleles (*n*)		Double-Gene-Deleted Alleles (*n*)	
b	.55	(100)	.57	(28)	1.0	(6)
D	.76		.60		1.0	
E	.68		.51		1.0	
h	.65		.72		1.0	
b/D/E	.41 (32)		.50 (20)		1.0	
b/D/E/F1	.31 (32)		.45 (20)		1.0	
b/D/E/F1/h[a]	.20 (32)		.45 (20)		1.0	

[a] $\chi^2 = 5.170$, df $= 1$, $P < .05$.

Discussion

The molecular basis of GALNS deficiency leading to the clinical symptoms of Morquio disease is of particular interest because of its allelic heterogeneity, clinical variability, and the presence of the common mutation characteristic for each ethnic population. In the present study we identified four new mutations (three missense mutations and one nonsense mutation) causing different clinical phenotypes in Japanese MPS IVA patients, who were compound heterozygotes with the double gene deletion on their other alleles (Hori et al. 1995). We also found evidence that this unique mutation is common in the Japanese population. Nonsense mutation (Q148X) will result in polypeptide chain termination and loss of the C-terminal portion, 375 amino acids of the polypeptide, which may lead to a complete destruction of GALNS activity, and therefore to a severe phenotype. Proline 151, which is changed to serine in patient 3 or leucine in patient 4, is conserved in all eukaryotic sulfatases, except iduronate-2-sulfatase (IDS; Wilson et al. 1990) (fig. 5). The amino acid proline is necessary for kink or β-turn in the secondary structure of the protein (Vanhoof et al. 1995). Therefore, the replacement of the proline 151 by serine or leucine will alter the secondary conformation, resulting in a change in the active structure of GALNS. The mutation of valine 138 to alanine in patient 1 occurred within the conserved amino acid sequence. Four kinds of sulfatases (GALNS, IDS, human arylsulfatase B [ARSB; Peters et al. 1990], and urchin arylsulfatase [ARSU]) show valine at this codon, while glucosamine-6-sulfatase (G6S; Robertson et al. 1988) and human arylsulfatase A (ARSA; Stein et al. 1989*b*) contain alanine at the same position. Valine 138 is the N-terminal amino acid of the heptapeptide 138-144 V/AGKWHLG conserved among the sulfatase family (Tomatsu et al. 1991). This peptide contains a long continuous stretch of amino acids thought to be involved in

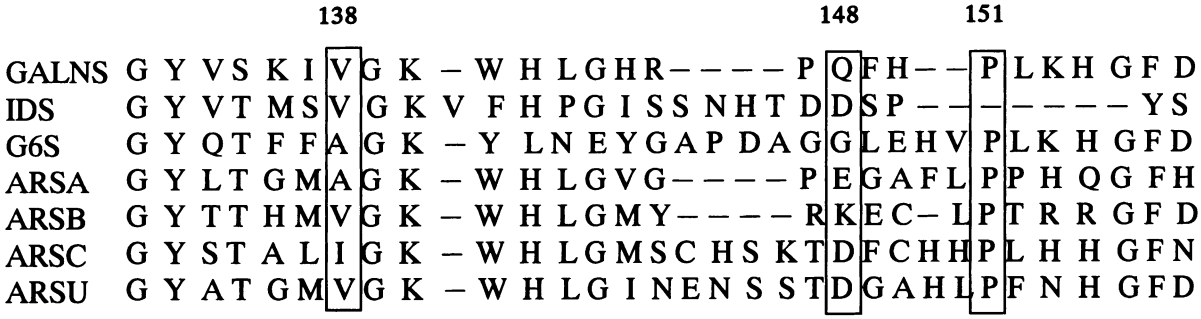


Figure 5 Partial amino acid sequences of human GALNS to IDS, G6S, ARSA, ARSB, ARSC (arylsulfatase C; Stein et al. 1989*a*), and ARSU (urchin arylsulfatase; Sasaki et al. 1988) with present mutations. Amino acid residues of the mutation site are boxed. Amino acid numbering of the mutation site is on the top, according to the number of the amino acid sequence of GALNS. Broken-lined sequences are unique to each sulfatase sequence.

assembly of active sites of these sulfatases. An alanine instead of a bulky valine will alter the stability of the secondary structure of the polypeptide in this region because of the lower free energy of transfer for alanine than for valine (Matsumura et al. 1988; Shortle et al. 1990). However, since alanine is present at this codon in some sulfatases, this V138A mutation might be expected to produce a milder phenotype. This expectation is consistent with the finding that the patient's clinical phenotype was an intermediate form, milder than the severe phenotype produced by homozygosity for the double-gene-deletion allele.

We used SSCP analysis to screen 60 other MPS IVA patients for the above mutations, followed by dot blot, restriction-enzyme analysis, and sequencing. V138A, Q148X, and P151S changes were each found only in a single family. Thus, these three mutations must be rare. However, the P151L mutation was also detected in Caucasian-Pakistan siblings in a homoallelic state (Tomatsu et al. 1995*b*). To date, various kinds of polymorphic sites have been detected in the GALNS gene. Haplotyping of control and MPS IVA patients revealed >15 different haplotypes by using four RFLPs (Iwata et al. 1995). Although genetic variability exists in both Japanese and Caucasian populations, the major common mutations in the GALNS gene in each racial group are absent in the other racial group.

Direct evidence for the four alterations being the disease-causing mutations was provided by transient expression in GALNS-deficient fibroblasts. Each mutant GALNS exhibited a significant reduction in catalytic activity. It is surprising that we found no significant difference in enzyme activity expressed transiently between the V138A mutation associated with milder-disease manifestations and three other mutations association with severe-disease manifestations. Although a different expression system might reveal differences in the activity and stability of the residual enzymes expressed by each mutant GALNS cDNA, our results demonstrate at least that these four substitutions impair GALNS activity and are the disease-causing mutations.

Identification of these point mutations in each family made accurate heterozygote detection feasible for relatives, using restriction enzymes or ASOs in addition to SSCP. A molecular diagnosis of heterozygotes is particularly valuable since there is significant overlap in enzymatic activities from various tissue sources for obligate heterozygotes and normal individuals.

We detected a common double deletion in six alleles (five patients), in all cases resulting from the same DNA rearrangement. The double gene deletion was observed only in Japanese MPS IVA patients, not in Caucasian patients. It is surprising that patients with a rare disease such as MPS IVA (e.g., occurring in <1/100,000 births in Japan) would share the same rare double gene deletion, when all the patients were nonconsanguineous and dispersed in a wide area of Japan. Possible explanations for this high prevalence of the double gene deletion in Japanese patients include the following: the double-deleted alleles are inherited from a common ancestor (founder effect); the region around the breakpoints of double gene deletion is particularly susceptible to deletion, leading to a recurring mutation; and high prevalence caused by selective pressure for the mutation.

Haplotype analysis of the mutant alleles with double gene deletion showed that all mutant alleles were associated with b/D/E/F1/h, except for some variation in a VNTR-like element. Moreover, although expanded haplotyping with a VNTR-like polymorphism showed that this common mutation was associated with four different haplotypes, one haplotype (b/D/E/F1/h/11.8 kb) was observed among three double-gene-deleted alleles. This variation could easily have arisen after the origin of the double deletion. The data above, together with the absence of this common mutation in Caucasians, strongly support a founder effect for the frequency

of double gene deletion on the GALNS gene in Japanese. Such consistent association between a specific mutation and a specific haplotype has been observed in several other genetic disorders (Polten et al. 1991; Svensson et al. 1991; Scott et al. 1992, Taroni et al. 1993, Zhang et al. 1993) and has usually been explained by a founder effect. The present study showed linkage disequilibrium between the normal individuals and MPS IVA patients for the haplotype of b/D/E/F1/h in association with this unique common deletion (table 1). After this haplotype, b/D/E/F1/h alleles associated with double gene deletion are subtracted from the number of MPS IVA alleles used in this study, and there is no longer disequilibrium. This implies that double gene deletion was the mutation responsible for the disequilibrium. Although most of the alleles of the haplotype accompany the diffusion of the double gene deletion, the wide haplotype diversity in the VNTR-like element suggests that double gene deletion is an old molecular defect.

Illegitimate recombinational events would be required to produce the double gene deletion, which are important causes of germinal rearrangements in several human inherited disorders. The following mechanism of the double gene deletion has been suggested: deletion 1 arose from a homologous recombination between Alu-Alu repetitive sequences containing the perfect direct repeat. Deletion 2 derived from an illegimate nonhomologous recombination with incomplete direct repeat matched with adequate modified mispairing system (Hori et al. 1995). This complex mutation occurred only in the original ancestor, and the VNTR variation appeared in subsequent generations. In order to attempt to identify a structural motif that might induce these events in Japanese patients, we determined the sequences around the suggested breakpoints in both normal Japanese and Caucasians. There was no difference of nucleotide sequences between the two populations. We conclude that this mutation occurred on one specific haplotype background in ancient times and distributed to the four haplotypes by recombination at the VNTR-like element. We know of no prior example of this complex type of common mutation on any disease-causing mutations.

To screen for the double gene deletion, we used a PCR method that provides an easy and rapid alternative to Southern blot analysis. Actual screening of other 67 MPS IVA patients (17 Japanese and 50 Caucasian) by this method yielded neither a 837-bp fragment from deletion 1 nor a 439-bp fragment from deletion 2 encompassing each breakpoint. Nonetheless, this method can provide prenatal diagnosis for at-risk pregnancies for future children of the five analyzed families, by using DNA from amniotic fluid or chorionic villus cells, and will facilitate studies to determine the true incidence of the double gene deletion among children with MPS IVA, in various races.

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