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Mucopolysaccharidosis VI (Maroteaux-Lamy Syndrome): Six Unique Arylsulfatase B Gene Alleles Causing Variable Disease Phenotypes

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Summary

Mucopolysaccharidosis type VI, or Maroteaux-Lamy syndrome, is a lysosomal storage disorder caused by a deficiency of the enzyme arylsulfatase B (ASB), also known as N-acetylgalactosamine-4-sulfatase. Multiple clinical phenotypes of this autosomal recessively inherited disease have been described. Recent isolation and characterization of the human ASB gene facilitated the analysis of molecular defects underlying the different phenotypes. Conditions for PCR amplification of the entire open reading frame from genomic DNA and for subsequent direct automated DNA sequencing of the resulting DNA fragments were established. Besides two polymorphisms described elsewhere that cause methionine-for-valine substitutions in the arylsulfatase B gene, six new mutations in six patients were detected: four point mutations resulting in amino acid substitutions, a 1-bp deletion, and a 1-bp insertion. The point mutations were two G-to-A and two T-to-C transitions. The G-to-A transitions cause an arginine-for-glycine substitution at residue 144 in a homoallelic patient with a severe disease phenotype and a tyrosine-for-cysteine substitution at residue 521 in a potentially heteroallelic patient with the severe form of the disease. The T-to-C transitions cause an arginine-for-cysteine substitution at amino acid residue 192 in a homoallelic patient with mild symptoms and a proline-for-leucine substitution at amino acid 321 in a homoallelic patient with the intermediate form. The insertion between nucleotides T1284 and G1285 resulted in a loss of the 100 C-terminal amino acids of the wild-type protein and in the deletion of nucleotide C1577 in a 39-amino-acid C-terminal extension of the ASB polypeptide. Both mutations were detected in homoallelic patients with the severe form of the disease. Expression of mutant cDNAs encoding the four amino acid substitutions and the deletion resulted in severe reduction of both ASB protein levels and arylsulfatase enzyme activity in comparison with a wild-type control. The six mutations described in the present study were unique among 25 unrelated mucopolysaccharidosis VI patients, suggesting a broad molecular heterogeneity of the Maroteaux-Lamy syndrome.

Introduction

Mucopolysaccharidosis type VI (MPSVI), or Maroteaux-Lamy syndrome, is a rare, autosomal recessively inherited glycosaminoglycan storage disease caused by the deficiency of the enzyme arylsulfatase B (ASB; E.C.3.1.6.1), also known as N-acetylgalactosamine-4-sulfatase. ASB is required for the degradation of the glycosaminoglycans dermatan sulfate and chondroitin 4-sulfate (Matalon et al. 1974; O'Brien et al. 1974). ASB deficiency causes intralysosomal accumulation and urinary secretion of large amounts of partially degraded dermatan sulfate. MPSVI patients may present with a varying spectrum of clinical phenotypes. At least three distinct ages at onset have been differentiated: a severe, infantile form is characterized by early onset (before 2 years of age) and rapid disease progression; an interme-

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diate or juvenile form demonstrates onset of disease in late childhood; and a mild or adult form demonstrates onset after the 2d decade. Disease progression in the juvenile and adult forms is typically slower than in the infantile form. Disease symptoms include growth retardation, coarse facial features, restriction of articular movement with development of claw hand, dysostosis multiplex, hepatosplenomegaly, aortic valvular dysfunction, and corneal clouding. In contrast to most other mucopolysaccaridoses, mental development in MPSVI is normal. Patients with the severe form of MPSVI often die of cardiopulmonary complications in their 2d or 3d decade of life (Neufeld and Muenzer 1989).

The isolation and characterization of human ASB cDNAs (Peters et al. 1990; Schuchman et al. 1990) has made possible the investigation of molecular lesions in the ASB gene of MPSVI patients. Seven ASB gene mutant alleles have been identified which cause different clinical phenotypes in five MPSVI patients (Wicker et al. 1991; Jin et al. 1992; Litjens et al. 1992; Arlt et al., in press). Since the structure of the wild-type human ASB gene was not available at the time these analyses were performed, ASB mRNA of the MPSVI patients was reverse transcribed and PCR amplified, and the sequence of the amplified cDNA fragments was determined either after subcloning or by direct sequencing of the amplified cDNA fragments. Recently, the structure of the human ASB gene comprising eight exons and interrupted by seven introns has been elucidated (Modaressi et al. 1993).

Knowledge of the intron/exon structure of the ASB gene and DNA sequences of the intron/exon boundaries made it possible to analyze exonic ASB mutations by PCR amplification of single exons from genomic MPSVI patient DNA and subsequent DNA sequence analysis without subcloning of the amplified DNA fragments. Using this experimental approach, we identified six mutant ASB gene alleles underlying different clinical phenotypes in six MPSVI patients.

Material and Methods

Material

Restriction enzymes and DNA modifying enzymes were from Boehringer Mannheim, New England Biolabs, Pharmacia, Beckman, or BRL. [α^{32}P]dCTP (specific activity 3,000 Ci/mmol), and [γ^{32}P]ATP (specific activity 3,000 Ci/mmol) were from Amersham. Dye Terminator Cycle Sequencing Kits were from Applied Biosystems, and Hybond N membrane was from Amersham.

Cell Lines and Cell Culture

MPSVI fibroblast lines of patients B.V., A.Mo., and L.E. were obtained from The Human Genetic Mutant Cell Repository, Lyons. Patient cell lines U.S., A.B., A.O., K.K., Z.A., Kn.K., M.K., and K.L. were provided by M. Beck, Mainz, Germany; cell lines P.R., M.T., S.S., P.P., C.H., and M.A. were provided by X. Krasnopolskaya, Moscow; cell line A.M. was provided by M. Niermeyer, Rotterdam; and cell line Y.V. was provided by R. Gitzelmann, Zurich. Cell lines M.Ak., P.A., D.H., M.O., W.D., and Ü.O. were from our own diagnostic samples. LTK$^-$ cells were from the American Type Culture Collection (Rockville, MD). Human fibroblasts and LTK$^-$ cells were maintained in culture as described elsewhere (von Figura et al. 1983). Fibroblast lines of patients U.S., A.B., A.O., M.Ak., A.M., and L.E. were used for sequence analysis of exonic mutations, and a brief description of these cases is given below.

Patients

Patient U.S. was the first child of consanguineous parents. At the age of 6 wk, the patient presented with an inguinal hernia on the left side and again at the age of 4 mo with an inguinal hernia on the right side. At the age of 1 year, he presented with pronounced coarse facial features, macrocephaly, claw hands, dysostosis multiplex, and insufficiencies of the bicuspidal and aortic valves. ASB enzyme activity was reduced severely, leading to a diagnosis of MPSVI, severe form.

Patient A.B. was the fourth child of consanguineous parents. At 4 years of age, the patient had a height of 90 cm, his weight was 12 kg, the head circumference was 50 cm, and he had corneal clouding. At the age of 9 years, MPSVI symptoms were more obvious, his height was 115.6 cm, the weight 21.2 kg, and the head circumference 52 cm. Coarse facial features and a barrel-shaped thorax were apparent. This patient's phenotype was the milder, juvenile form of MPSVI.

Patient A.O. was the first child of consanguineous parents. At 18 mo of age, the patient had a height of 85 cm, his weight was 13.2 kg, and the circumference of his head was 52.8 cm. His facial features were moderately coarse, and the nasal root was flattened. He had a short trunk with thoracolumbal kyphosis and presented with an umbilical hernia and bilateral inguinal hernias. The patient's phenotype was MPSVI, intermediate form.

Patient M.Ak. was the second child of unrelated parents (Van Biervliet et al. 1977). At the age of 5 mo, the patient presented with bilateral inguinal hernias, a barrel-shaped thorax, restricted movement of the elbows, and mild corneal clouding. During 7 mo of observation MPSVI symptoms progressed rapidly, and a diagnosis of MPSVI, severe form, was made.

Patient A.M. was the sixth child of consanguineous parents. At the age of 9 mo, he presented with multiple skeletal deformations, including radius, metacarpals, and phalanges; a pronounced lumbar kyphosis; bilateral coxa valga; macrocephaly; and diffuse corneal clouding. This patient's phenotype was MPSVI, severe form.

Patient L.E. was the seventh child of consanguinous parents. Diagnosis of MPSVI was confirmed at the age of 14 mo by detection of severely reduced ASB activity. At the age of 5 years 9 mo he presented with coarse facial features, short stature, lumbar kyphosis, restricted joint movement, claw hands, and corneal clouding. This patient's phenotype was MPSVI, severe form.

Preparation of Genomic DNA, PCR Amplification, and DNA Sequencing

Genomic DNA from normal and MPSVI fibroblasts was prepared as described elsewhere (Strauss 1990). Exons 1–8 of the ASB gene were PCR amplified from genomic patient DNA on eight DNA fragments, each of which contained one exon (exons 2–7) or the coding region of one exon (exons 1 and 8). PCR amplifications were performed on a Perkin Elmer Cetus PE9600 thermocycler, utilizing the following primer pairs: exon 1, AB92 (5′-TTCCTCATTCTATCAGCGGTACAAG-3′) and AB91 (5′-GAGAAGCCGCCGGGACCCATAACT-3′); exon 2, AB39 (5′-ATTTTATGTTTATCTCTGTAAG-3′) and AB40 (5′-AAAGAAACATGTGCATTTCC-3′); exon 3, AB56 (5′-TGCTTCCATTCTTGC-3′) and AB49 (5′-GTAAATAGAAGCAAAACTT-3′); exon 4, AB74 (5′-CCTTCTATATTTAATGCTTCAATATCC-3′) and AB73 (5′-CTAGCTTTGCCAAGAGATGATTTTCC-3′); exon 5, AB44 (5′-CATCATCCTCATGCC-3′) and AB45 (5′-GAAAAAGGGCAGGGTGT-3′); exon 6, AB59 (5′GCGCGAATTCCCTTAAAAATTGTTTTCC-3′) and AB51 (5′-TAGCAATGCACTGGTACTC-3′); exon 7, AB60 (5′-TCAAACTATTTCTTCC-3′) and AB52 (5′-TTGCTAAGCTAAGGACTCT-3′); and exon 8, AB61 (5′-GCGCGAATTCTGCTCAGTAAACTGT-3′) and AB68 (5′-GAAAAGGCCTGAGGTCCAACTTCC-3′). Amplification primers were removed by filtration through Centricon-100 (Amicon) membranes, and PCR-amplified DNA fragments were subsequently sequenced directly without subcloning, by fluorescent dye terminator cycle sequencing and analysis on an automated DNA sequencer (Applied Biosystems). Coding- and noncoding-strand amplification primers listed above were used as sequencing primers for analysis of the coding and noncoding DNA strand, respectively. For sequence analysis of exons 1 and 8, internal oligonucleotide primers were employed as well (sequences for these primers were deduced from Peters et al. [1990] and Modaressi et al. [1993]).

Construction of Mutant ASB Expression Plasmids and Stable Transfection of LTK⁻ Cells

The wild-type ASB cDNA (Peters et al. 1990) was subcloned into M13mp18, and in vitro mutagenesis was performed with mutant allele–specific oligonucleotides corresponding to mutations G144R, C192R, L321P, and C521Y, by the method of Nakamaye and Eckstein (1986). The mutation ΔC1577 was introduced into the wild-type ASB cDNA by PCR amplification using a noncoding PCR primer corresponding to the mutant allele DNA sequence. The mutations were introduced into the eukaryotic expression vector pBEH (Artelt et al. 1988) by subcloning DNA restriction fragments from the M13mp18 clones carrying the respective mutation into the wild-type ASB expression plasmid pCA7 (Peters et al. 1990). DNA sequences of the resulting mutant ASB expression plasmids pCA7-G144R, pCA7-C192R, pCA7-L321P, pCA7-C521Y, and pCA7-ΔC1577 were confirmed for the entire subcloned mutant M13mp18 DNA fragments.

Mutant ASB expression plasmids were cotransfected with the resistance plasmid pSV2neo (Southern and Berg 1982) into LTK⁻ cells by the calcium phosphate method (Wigler et al. 1977). Two days after transfection, the medium was supplemented with 0.5 mg geneticin (G418)/ml. Pools of stably transfected cells were assayed for arylsulfatase activity and human ASB–specific mRNA, as described below.

Isolation of Total RNA, Northern and Western Blot Analyses, and Detection of Arylsulfatase Enzyme Activity

Total RNA was prepared from wild-type (Wicker et al. 1991) and mutant ASB-expressing LTK⁻ cells, as well as from untransfected LTK⁻ cells as described elsewhere (Chirgwin et al. 1979). Ten micrograms of total RNA in 20 μl of 20-mM morpholinopropane sulfonic acid (MOPS) pH 6.8, 6% formaldehyde, and 66% form-

amide was heated to 70°C for 10 min and electrophoresed through a 1.3% agarose gel containing 0.7% formaldehyde in 20 mM MOPS pH 6.8 and subsequently transferred to Hybond-N membranes. For northern blot analysis, filters were hybridized to a human ASB cDNA probe (Peters et al. 1990) and to a 280-bp cDNA fragment from murine glyceraldehyde-3-phosphate dehydrogenase (G3PD; Lyons et al. 1989). Hybridization and washing of filters were performed as described by Peters et al. (1990). RNA signals were quantified by densitometric scanning, and the amount of RNA from wild-type and mutant human ASB-expressing cells was normalized to the amount of RNA in wild-type ASB-expressing cells (e.g., see the wild-type RNA in fig. 2) using the G3PD signals. Subsequently, levels of ASB enzyme activities were normalized to wild-type ASB-expressing cells with the normalized ASB mRNA levels.

For western blot analysis, 80 μg of protein from homogenates of expressing LTK^- cells were separated in 10% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes. Membranes were blocked overnight at 4°C in PBS-T (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7\ H_2O$, 1.4 mM KH_2PO_4 pH 7.3, 0.05% Tween 20) containing 5% low-fat milk powder. Membranes were washed for 1 × 15 min and 2 × 5 min in PBS-T and were incubated with affinity-purified ASB-specific antiserum (20 μg/ml) in PBS-T for 1 h at room temperature. Subsequently, membranes were washed 3 × 15 min in PBS containing 2% SDS at room temperature and 2 × 5 min in PBS-T and were incubated with peroxidase-conjugated goat anti-rabbit IgG (H+L; Dianova) in a 1:50,000 dilution in PBS-T for 30 min at room temperature. The final wash was 1 × 15 min and 4 × 5 min in PBS-T. Blots were developed with an enhanced chemiluminescence system (ECL; Amersham). Exposure times were 1–3 min.

Arylsulfatase activities were measured in homogenates of ASB-expressing LTK^- cells and untransfected LTK^- cells prepared in 10 mM Tris/HCl pH 7.4, 150 mM NaCl, and 0.1% Triton X-100. Homogenate (10–50 μl) was incubated with 120 μl of 10-mM p-nitrocatechol sulfate in 0.5 M sodium acetate pH 5.5 for 10–30 min at 37°C. The reaction was stopped by the addition of 300 μl of 0.6 N NaOH, and absorbance was measured at 510 nm (Allen and Roy 1968; Steckel et al. 1983). One unit is the amount of enzyme catalyzing the formation of 1 μmol of p-nitrocatechol/min. Protein concentration was determined by the method of Lowry et al. (1951). Specific arylsulfatase enzyme activities were calculated and multiplied with the normalizing factor for the ASB mRNA expression level (see above) to allow direct comparison of the ASB activities determined for wild-type and mutant ASB-expressing cells (listed in fig. 2).

Results

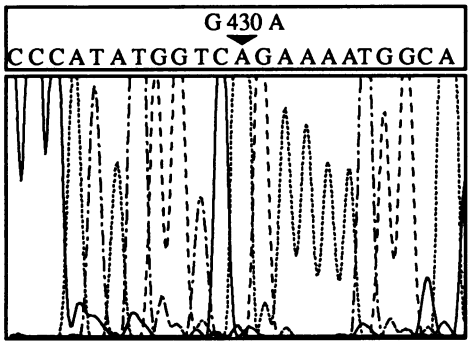
Identification of Exonic ASB Gene Mutations in Six MPSVI Patients

The open reading frame of the ASB gene was PCR amplified from genomic patient DNA on eight DNA fragments, each representing a complete exon (exons 2–7) or the translated region of an exon (exons 1 and 8). After removal of the amplification primers, the sequence of the coding and the noncoding strand of each DNA fragment was determined directly by an automated, nonradioactive sequencing method (for details, see Material and Methods). By using this approach the nucleotide sequence of the entire open reading frame was determined for six MPSVI patients presenting different clinical phenotypes.

In patient A.M., a G-to-A transition at nucleotide 1126 and, in patients A.B. and A.O., a G-to-A transition at nucleotide 1072 (numbering according to Peters et al. 1990) were detected. In all three cases these alterations should be present on both alleles, since these patients have a consanguineous background (data not shown). Both transitions resulted in methionine-for-valine substitutions and have been described as polymorphisms (Jin et al. 1991; Wicker et al. 1991).

Four nucleotide exchanges detected in four different patients—two G-to-A transitions at nucleotides 430 (patient U.S.; fig. 1*A*) and 1562 (patient M.Ak.; fig. 1*D*) and two T-to-C transitions at nucleotides 574 (patient A.B.; fig. 1*B*) and 962 (patient A.O.; fig. 1*C*)—resulted in amino acid substitutions. In two of these cases, cysteine residues were substituted by arginine (C192R; patient A.B., mild disease phenotype) or by tyrosine (C521Y; patient M.Ak., severe form). In the other two cases, the nucleotide alterations resulted in an arginine-for-glycine substitution (G144R; patient U.S., severe phenotype) and a proline-for-leucine exchange (L321P; patient A.O., intermediate form; also see fig. 1). Patients U.S., A.B., and A.O. can be considered homoallelic, since they have a consanguineous background. Since the parents of M.Ak. are not related, it is unlikely that M.Ak. is homoallelic. The second allele in this patient may have escaped attention because of a deletion including the mutant site on exon 8 or a mutation in an oligonucleotide hybridization site. Therefore the

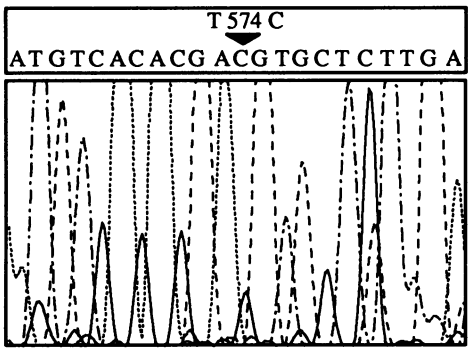
A



patient: U.S.
diagnosis: MPS VI, severe
mutation: G 144 R

w.t. ATG GTC GGA AAA TGG
142 Met Val Gly Lys Trp
Arg
m.t. ATG GTC [A]GA AAA TGG
430

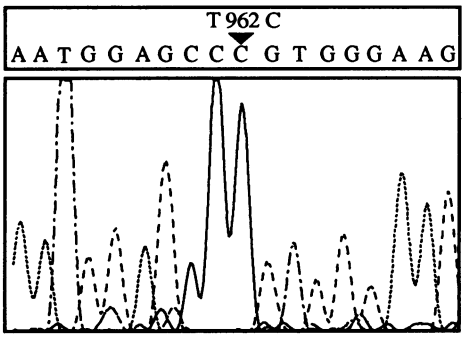
B



patient: A.B.
diagnosis: MPS VI, mild
mutation: C 192 R

w.t. ACA CGA TGT GCT CTT
190 Thr Arg Cys Ala Leu
Arg
m.t. ACA CGA [C]GT GCT CTT
570

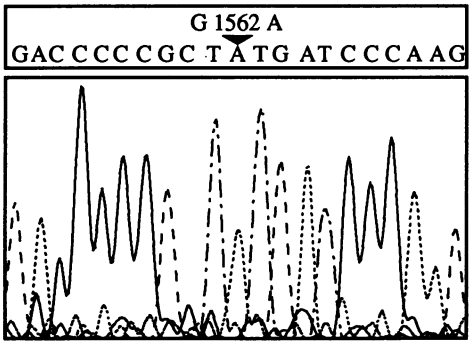
C



patient: A.O.
diagnosis: MPS VI, intermediate
mutation: L 321 P

w.t. TGG AGC CTG TGG GAA
319 Trp Ser Leu Trp Glu
Pro
m.t. TGG AGC C[C]G TGG GAA
960

D



patient: M.Ak.
diagnosis: MPS VI, severe
mutation: C 521 Y

w.t. CCC CGC TGT GAT CCC
519 Pro Arg Cys Asp Pro
Tyr
m.t. CCC CGC T[A]T GAT CCC
1560

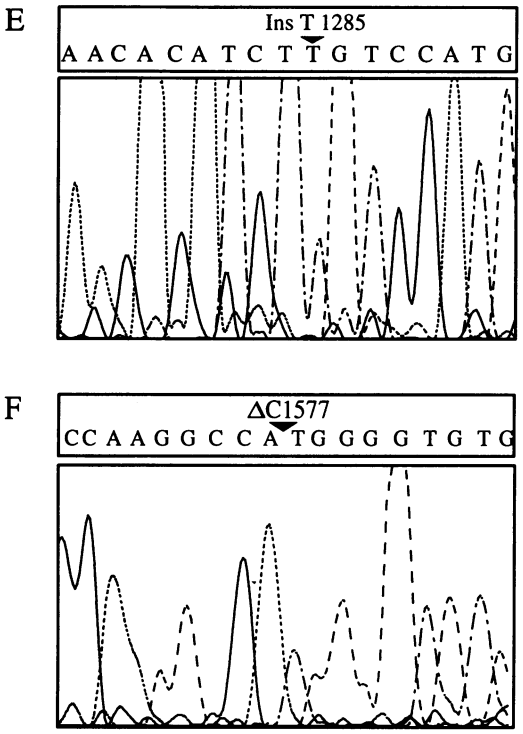


Figure 1 Identification of point mutations, a deletion, and an insertion in ASB-gene exons of six MPSVI patients presenting different clinical phenotypes. The open reading frame of the ASB gene from six MPSVI patients with different clinical phenotypes was PCR amplified on eight DNA fragments. Coding and noncoding strands of the amplified DNA fragments were sequenced directly by fluorescent dye terminator cycle sequencing and analysis on an automated DNA sequencer (see Material and Methods). Sequencing diagrams covering positions of nucleotide alterations are shown. The nucleotide sequence is given above the graphs; nucleotide alterations in comparison with the wild-type ASB cDNA sequence (Peters et al. 1990) are marked with an arrowhead, and each nucleotide substitution (panels *A–D*), nucleotide insertion (panel *E*), or nucleotide deletion (panel *F*) detected is given above the arrowhead. In the right half of each panel, wild-type (w.t.) and mutant (m.t.) nucleotide and amino acid sequences are given (amino acid numbering is on the left, and nucleotide numbering is below the mutant sequences, both according to Peters et al. 1990). Patient initials, diagnosis with clinical phenotype, and abbreviation of the respective mutation are listed.

presented data do not exclude heteroallelism in this patient (fig. 1).

The two other mutations detected were a 1-bp insertion between nucleotides T1284 and G1285 in patient A.M. (InsT1285; fig. 1*E*) and a 1-bp deletion of nucleotide C1577 in patient L.E. (ΔC1577; fig. 1*F*). Since these two patients, who exhibited a severe disease phenotype, have a consanguineous background, they can also be considered homoallelic. The insertion results in a premature translational stop codon six triplets 3′ of the alteration and hence a truncated protein, which is 100 amino acids shorter than the wild-type protein. The deletion leads to a substitution of the C-terminal 7 amino acids of the wild-type ASB polypeptide with a 39-amino-acid C-terminal extension of the encoded mutant polypeptide.

Independent Verification of Mutant Alleles

For independent verification of the detected DNA sequence alterations, the respective PCR DNA fragment, as well as the corresponding DNA fragment of 19 unrelated MPSVI patients, was slot blotted and hybridized to a mutant allele-specific and a corresponding wild-type-specific oligonucleotide. In all six cases the mutant oligonucleotides hybridize to the DNA fragment of the respective MPSVI patient, whereas the wild-type oligonucleotides hybridize to DNA fragments of the 19 unrelated MPSVI patients (data not shown). We conclude that patients U.S., A.B., A.O., A.M., and L.E. are indeed homoallelic for the respective exonic sequence alteration and that the second allele in patient A.Mk. has escaped detection because of either a deletion of exon 8 or a failure to amplify this

allele because of a mutation in one of the oligonucleotide primer hybridization sites. None of the six mutant alleles described in this study is present in any of the 19 unrelated MPSVI patients investigated (data not shown).

Mutations Identified Reduce ASB Enzyme Activity to Background Levels

To investigate the effect of the four amino acid substitutions and the C-terminal extension (see fig. 1*A–D* and *F*) on the enzymatic activity of the encoded ASB polypeptides, the respective nucleotide alterations were introduced into the wild-type ASB expression plasmid pCA7 (Peters et al. 1990) either by site-directed in vitro mutagenesis or by PCR amplification using mutant oligonucleotides (for details, see Material and Methods). The resulting mutant expression plasmids pCA7-G144R, pCA7-C192R, pCA7-L321P, pCA7-C521Y, and pCA7-ΔC1577 were each cotransfected into mouse LTK$^-$ cells with the plasmid pSV2neo, conferring resistance to G418. After selection, homogenates of cell pools were assayed for arylsulfatase enzyme activity by using the artificial substrate p-nitrocatechol sulfate. The specific arylsulfatase activity per milligram of total protein was determined and normalized to the ASB mRNA level of wild-type ASB-expressing cells (see W.T. in fig. 2). Toward this end, the levels of human ASB mRNA and mouse glyceraldehyde-3-phosphate dehydrogenase mRNA were determined in a northern blot, which was first probed with human ASB cDNA (Peters et al. 1990) and was subsequently hybridized to a mouse glyceraldehyde-3-phosphate dehydrogenase cDNA probe (Lyons et al. 1989). The arylsulfatase activities determined for wild-type and mutant ASB-expressing cells show that the enzymatic activities of all five mutant ASB polypeptides investigated are severely reduced or even not above the level of untransfected LTK$^-$ cells at all (fig. 2). Furthermore, western blot analysis of homogenates prepared from wild-type and mutant ASB-expressing cells revealed a steady-state level of ASB polypeptide, which was below the level of detection in all five mutant ASB-expressing cells (fig. 2). Even after longer exposure, no ASB-specific protein was detectable in the mutant ASB-expressing cells (data not shown).

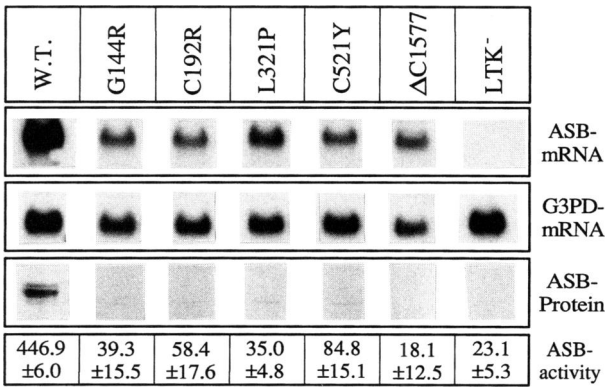


Figure 2 ASB-mRNA and -protein levels and ASB activity in LTK$^-$ cells stably transfected with wild-type and mutant ASB-encoding expression plasmids. Homogenates of LTK$^-$ cells stably expressing wild-type human ASB (W.T.) or the ASB mutant polypeptides G144R, C192R, L321P, C521Y, or ΔC1577 and homogenates of untransfected LTK$^-$ cells were analyzed for human ASB-protein levels and assayed for arylsulfatase activity. Enzyme activities are expressed as nanomoles of p-nitrocatechol sulfate cleaved per minute and milligrams of protein, and activities for mutant ASB-expressing cells were normalized to the ASB-mRNA expression level of wild-type ASB-expressing cells ± SD; $n=3$ for wild-type ASB; and $n=5$ for mutant ASB-expressing cells and untransfected LTK$^-$ cells. Normalization factors were calculated from human ASB and mouse G3PD-mRNA levels, determined by northern blot analysis of 10 μg total RNA isolated from wild-type and mutant ASB-expressing cells and subsequently hybridized to an ASB (ASB-mRNA) and a G3PD (G3PD-mRNA) probe (also see Material and Methods).

Discussion

In this study, we identified six ASB-gene mutant alleles causing different clinical phenotypes of MPSVI in six patients. Four of the identified mutations are nucleotide exchanges in the open reading frame of the ASB gene, causing amino acid substitutions. The fifth mutation is a 1-bp insertion resulting in a premature stop codon and consequently a deletion of the C-terminal 20% of the ASB polypeptide. The sixth mutation is a 1-bp deletion near the 3′ end of the open reading frame, causing a 39-amino-acid extension of the mutant ASB polypeptide. Five of the six patients are likely to be homoallelic, since their parents are consanguineous. One patient, M.Ak., whose parents are unrelated, may be heteroallelic, even though only one mutation was detected.

The characterization of the human ASB gene and the establishment of the DNA sequences at the exon-intron borders (Modaressi et al. 1993) greatly facilitated the mutational analysis in MPSVI patients. Conditions for PCR amplification of the entire open reading frame of the ASB gene on eight DNA fragments, each covering either an entire exon or—in the cases of the first and the last exon—the ASB polypeptide coding seg-

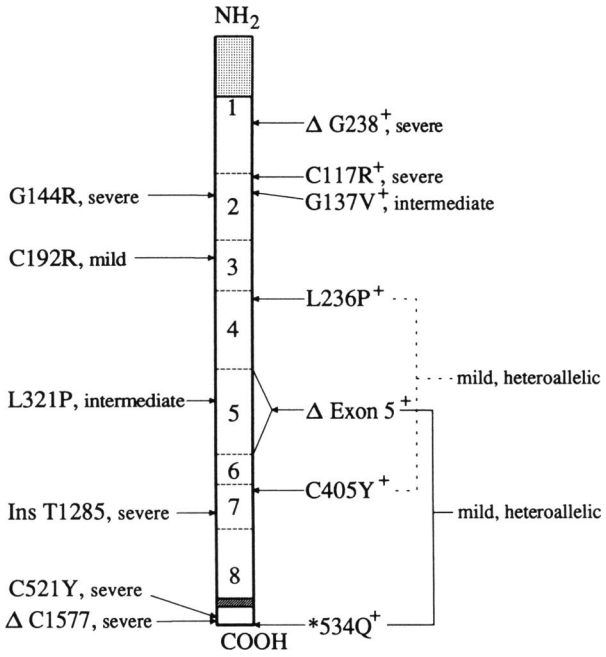


Figure 3 Map of hitherto identified ASB-gene mutations. The wild-type ASB polypeptide is represented from N-terminus (NH_2) to C-terminus (COOH); the stippled box represents the N-terminal leader peptide; the hatched box represents peptide excised by intralysosomal processing; unblackened boxes represent 53-kD and 8-kD mature ASB polypeptides; the segments of polypeptide are encoded by the ASB gene exons 1–8, as indicated by numbers and dashed lines. Mutations marked "+" were described elsewhere: G137V by Wicker et al. (1991), C117R, L236P, and C405Y by Jin et al. (1992), ΔG238 by Litjens et al. (1993), and ΔExon 5 and *534Q by Arlt et al. (in press). Mutations listed on the left were identified in this study. Two patients were heteroallelic: L236P/C405Y and ΔExon5/*534Q, as indicated by the brackets. The clinical phenotype is given behind each mutation or pair of mutations.

ments of an exon, and procedures for direct, automated DNA sequence analysis were developed. This approach reduces the number of sequence reactions necessary for the analysis of one exon to two to four, in comparison with at least four sequence reactions for each DNA strand for exclusion of *Taq* polymerase errors when subcloned DNA fragments are used (Wicker et al. 1991). Furthermore, this method does not include time-consuming RNA isolation and reverse transcription to cDNA. On the other hand, only exonic mutations can be detected. Deletions of entire exons on one allele or changes in the 5′ and 3′ UTRs, as well as the promotor of the ASB gene, will escape detection. Indeed, we were not able to detect the molecular lesions in two documented MPSVI patients; in these two patients all exonic sequences were detectable and were found to be identical with the wild-type ASB gene sequences (data not shown). In the case of the potentially heteroallelic patient M.Ak., a putative second mutant allele may have as well been missed because of these methodological reasons.

For five of the six sequence alterations detected, it was shown by introduction into the wild-type ASB cDNA and stable expression in LTK^- cells that each of them causes a severe reduction of the ASB enzyme activity. In one of the cases investigated, the arylsulfatase activity was not above the level of endogenous arylsulfatase activity of LTK^- cells. The insertion T1285 was not expressed, since the loss of one-fifth of the C-terminal of the wild-type ASB polypeptide makes it very unlikely that this truncated mutant ASB polypeptide contributes any arylsulfatase activity.

Recently, a 50-amino-acid C-terminal extension of the ASB polypeptide in an MPSVI patient with intermediate phenotype caused by mutation of the translational stop codon to a glutamine codon was shown to cause degradation of the majority of newly synthesized protein before delivery to the Golgi bodies. It is interesting that this mutant ASB polypeptide exhibits a higher catalytic efficiency toward a natural substrate than does the wild-type ASB protein (Arlt et al., in press). The ΔC1577 causes a comparable C-terminal extension of the open reading frame by 39 amino acid codons and may have a similar effect on half-life and catalytical properties as that of the 50-amino-acid extension.

Two of the four amino acid substitutions described in the present study involve amino acids, which may be of special importance to the ASB polypeptide. Glycerine 144, which is changed to arginine in patient U.S. (fig. 1*A*) is conserved in all eukaryotic arylsulfatases, and it is the N-terminal amino acid of the hexapeptide 144-GKWHLG-149 conserved between ASB, arylsulfatase A, steroid sulfatase, and sea urchin arylsulfatase (Peters et al. 1990; Wilson et al. 1990). This hexapeptide is the longest contiguous stretch of amino acids conserved among these sulfatases, suggesting a major importance for the enzyme activity and/or the structure of these proteins. The mutation of cysteine 521 to tyrosine in patient M.AK. (fig. 1*D*) is the only cysteine residue in the 8-kD ASB polypeptide generated by intralysosomal proteolytic processing of the 64-kD ASB precursor to mature ASB composed of a 43-kD and the 8-kD polypeptide covalently linked by a disulfide bridge (Steckel et al. 1983; Litjens et al. 1991). When cysteine 521 is mutated to tyrosine, the disulfide bond between the

two polypeptides of mature ASB cannot exist and is likely to cause a major alteration of the overall structure of the enzyme.

Two other amino acid substitutions involve amino acids that are not conserved between the arylsulfatases. It remains to be determined whether substitution of cysteine 192 by an arginine residue interferes with a disulfide bond. It is also unclear whether the proline-for-leucine substitution at position 321 could have a major impact on the secondary structure of the mutant polypeptide, since the amino acid proline is known to be a residue capable of interrupting helical protein structures.

It is of interest that all 13 known ASB-gene mutant alleles (listed in fig. 3) have so far been detected only in single MPSVI patients. This observation underlines the wide genetic heterogeneity of the Maroteaux-Lamy syndrome.

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