Am. J. Hum. Genet. 50:795–800, 1992

Mucopolysaccharidosis Type VI: Identification of Three Mutations in the Arylsulfatase B Gene of Patients with the Severe and Mild Phenotypes Provides Molecular Evidence for Genetic Heterogeneity

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Summary

Mucopolysaccharidosis type VI (MPS VI; Maroteaux-Lamy disease) results from the deficient activity of the lysosomal enzyme, arylsulfatase B (ASB; *N*-acetylgalactosamine-4-sulfatase E.C.3.1.6.1). The enzymatic defect leads to the accumulation of the glycosaminoglycan, dermatan sulfate, primarily in connective tissue and reticuloendothelial cell lysosomes. Although MPS VI patients have normal intelligence and no neurologic abnormalities, the disease is clinically heterogeneous: severely affected individuals expire in childhood or early adolescence while those with the mild or intermediate phenotypes have a slower, milder disease course and a longer life span. The recent isolation of the full-length cDNA-encoding human ASB permitted an investigation of the molecular lesions underlying the phenotypic heterogeneity in MPS VI. The ASB cDNA-coding sequences were determined from two unrelated MPS VI patients with the severe (proband 1) and mild (proband 2) phenotypes. These patients had about 2% and 7% of normal ASB activity in cultured fibroblasts, respectively. Proband 1 was homoallelic for a T-to-C transition in nucleotide (nt) 349, which predicted a cysteine-to-arginine substitution in the ASB polypeptide at residue 117 (C117R). Proband 2 was heteroallelic, having a T-to-C transition in nt 707, which predicted a leucine-to-proline replacement at ASB residue 236 (L236P), and having a G-to-A transition in nt 1214, which predicted a cysteine-to-tyrosine substitution at ASB residue 405 (C405Y). These mutations did not occur in three other unrelated MPS VI patients or in 120 ASB alleles from normal individuals, indicating that they were not polymorphisms. The identification of these three ASB mutations documents the first evidence of molecular heterogeneity in MPS VI and provides an initial basis for genotype/phenotype correlations in this lysosomal storage disease.

Introduction

Mucopolysaccharidosis type VI (MPS VI; Maroteaux-Lamy disease) is a connective-tissue disorder that results from the deficient activity of the lysosomal sulfatase, arylsulfatase B (ASB; *N*-acetylgalactosamine-4-sulfate sulfatase, E.C.3.1.6.1) and from the accumulation of its glycosaminoglycan substrate, dermatan sulfate (Maroteaux et al. 1963; Stumph et al. 1973; Fluharty et al. 1975; Neufeld and Muenzer 1989). The disease is inherited as an autosomal recessive trait, and individuals affected with the severe form have a Hurler-like facial dysmorphia, short stature, marked dysostosis multiplex, hepatosplenomegaly, cardiomegaly, corneal clouding, and normal intelligence Death usually results in childhood or adolescence because of cardiopulmonary complications. In contrast, patients with the mild and intermediate forms of the disease have less severe manifestations and often survive into the seventh or eighth decade of life (Quigley and Kenyon 1974; Wald and Schmidek 1984).

Received October 14, 1991; revision received November 26, 1991.

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0002-9297/92/5004-0015$02.00

ASB hydrolyzes the 4-sulfate groups from the *N*-acetylgalactosamine residues present on dermatan sulfate (Allen and Roy 1968; Gnoit-Szulzychka 1972; Matalon et al. 1974). This lysosomal sulfatase has been purified from various sources (e.g., Gnoit-Szulzychka 1972; Shapira and Nadler 1975; McGovern et al. 1982; Gibson et al. 1987), and in human placenta and liver it exists as a monomer that may be dissociated into subunits of about 43 and 13 kDa by using sulfhydryl reducing reagents. Biosynthetic studies performed in cultured cells have shown that ASB is synthesized as a 64-kDa precursor polypeptide that is phosphorylated and proteolytically processed into the mature form (Steckel et al. 1983). Studies of the residual ASB activity and enzyme protein in cultured cells from unrelated MPS VI patients also have been reported (e.g., in Humbel 1976; Kolodny and Mumford 1976; Hopwood et al. 1986; Brooks et al. 1991). Using a variety of artificial (Humbel 1976; Kolodny and Mumford 1976) and natural (Hopwood et al. 1986) substrates, researchers have shown that the ASB activities in cultured fibroblasts from severe and mild MPS VI patients generally have less than 10% of normal activity. However, the amount of residual ASB activity does not reliably correlate with the degree of clinical severity, presumably because of difficulties in measuring low levels of enzymatic activity. Brooks et al. (1991) recently evaluated the ASB cross-reactive immunologic material (CRIM) levels in cultured fibroblasts from 16 unrelated MPS VI patients with the mild, intermediate, and severe phenotypes, by using a monoclonal antibody–based immunoquantification assay. It is interesting that all of these patients had less than 10% of normal ASB protein, and the most severely affected patients had slightly less CRIM than did the patients with the mild or intermediate phenotypes. These results indicated that clinical severity may be correlated with the amount of residual enzyme protein.

The recent isolation and characterization of a full-length cDNA-encoding human ASB (Peters et al. 1990; Schuchman et al. 1990) has facilitated the investigation of the molecular mutations causing MPS VI. The cDNA was 2,811 bp and encoded a precursor polypeptide of 533 amino acids. In addition, a common polymorphism in nt 1072 was identified in the ASB coding sequence (Jin et al. 1991), permitting identification of both alleles in informative individuals. In the present communication, the first mutations in the ASB gene that cause MPS VI are described for two unrelated MPS VI patients, one affected with the severe form and the other affected with a milder form. (During the review of the manuscript of the present paper, a point mutation in the ASB gene causing an intermediate MPS IV phenotype [G137V] was reported [Wicker et al. 1991]). These point mutations provide direct molecular evidence of the genetic heterogeneity underlying the mild and severe forms of MPS VI.

Material and Methods

Cell Lines and Patient Materials

Primary fibroblast cultures were established from skin biopsies obtained with informed consent from normal individuals (cell lines MS-1 and MS-2) and from five unrelated MPS VI patients (cell lines MS-3–MS-7). Cell line MS-5 was previously submitted to the Human Genetic Mutant Cell Repository, Camden, NJ (Repository number GM00519). The biochemical diagnosis of MPS VI was based on the demonstration of markedly deficient ASB activity in cultured cells and excess urinary dermatan sulfate excretion. Proband 1 (cell line MS-3), a 9-year-old white male of English/Irish ancestry on both sides of his family, had a severe form of MPS VI. At 9 years of age he was 39 inches tall (<5th percentile) and weighed 41 pounds (<5th percentile). He had severe dysostosis multiplex, the Hurler-like facial dysmorphia, macrocephaly, corneal opacities, and hepatosplenomegaly. He was intellectually normal but had decreased motor and sensory function in an L5 distribution. He was unable to move his feet but did have some flexion-extension ability at the knees and the hips, permitting him to walk with the aid of leg braces. Proband 2 (cell line MS-4), was a white male of European ancestry who had mild MPS VI. He was diagnosed at age 5 years, when a mild cardiac murmur and bilateral hearing loss were detected. At that time, mild dysostosis multiplex was noted. At age 21 years, he was short (57 inches), had mild left-ventricular hypertrophy, a slightly enlarged liver, no corneal opacities, and mild skeletal abnormalities. He walked without assistance and had substantial flexion-extension at the knees and the hips.

Enzyme and Protein Assays

Arylsulfatase A (ASA) and ASB activities were determined in cultured fibroblasts obtained from the MPS VI patients and from normal individuals by using the colorimetric substrate, *p*-nitrocatechol sulfate (McGovern et al. 1981). For these assays, ~2–

3×10^6 cultured fibroblasts were grown in 75-mm^2 culture flasks and were harvested with a rubber policeman at midconfluency in 10 mM Tris-HCl, pH 7.5. Following centrifugation at 3,000 *g*, the pellets were washed twice, resuspended in 1 ml of the same buffer, and lysed by sonication at 4°C in a cup sonicator (Heat Systems-Ultrasonics, Farmingdale, NY). Cell extracts were centrifuged (10,000 *g*) at 4°C, and 200 μl of each supernatant was used for each assay. One unit of enzymatic activity equals the amount of enzyme that hydrolyzes 1 nm of substrate/h at 37°C. Protein determinations were performed using a Biorad (Richmond, CA) protein determination kit according to the manufacturer's instructions.

cDNA Amplification and Sequencing

To amplify the ASB cDNA-coding sequences, total RNA was isolated from the cultured cells by standard procedures (Sambrook et al. 1989). First-strand cDNA was synthesized from ~10 μg of total RNA by using a cDNA synthesis kit according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis). The ASB-coding region was amplified in two overlapping fragments by PCR using *Taq* polymerase (Promega, Madison, WI) (Saiki et al. 1988). Sense and antisense PCR primers were constructed on an Applied Biosystems (Foster City, CA) model 380B DNA synthesizer (Itakura et al. 1984). Primers P1 (5′-AGACGACCTAGGCTGGAA-3′) and P2 (5′-GCTTCAAGTATTCCTCA-3′) were used to amplify a 604-bp product from the 5′ end of the ASB cDNA. The 3′ ASB-coding sequence was amplified in an 897-bp fragment by using sense and antisense PCR primers P3 (5′-AGGAATACTTGAAGCCA-3′) and P4 (5′-GTCCAACTTCCAATTGA-3′), respectively. Following amplification, the PCR products were isolated from agarose gels, were subcloned into the Bluescript KS (+) vector (Stratagene, La Jolla, CA), and were sequenced in both orientations by the dideoxy chain-termination method (Sanger et al. 1977). For each PCR product, at least 8–10 subclones were sequenced.

Dot-Blot Hybridization

To confirm the authenticity of the C117R and L236P mutations, genomic DNA from probands 1 and 2 was PCR amplified for dot-blot hybridization. The PCR primers and allele-specific oligonucleotides (ASOs) were synthesized as described above (Itakura et al. 1984). For the C117R mutation, a 130-bp fragment was amplified using sense and antisense PCR primers P5 (5′-CCGTACAGGTTTACAGC-3′) and P6 (5′-ACATTCCCAGGTGCCAT-3′), respectively. For the L236P mutation, a 64-bp fragment was amplified using sense and antisense PCR primers P7 (5′-AGCCTCTGTTTCTCTAC-3′) and P8 (5′-GTATTCCTCAGGGACCT-3′), respectively. Following amplification, dot-blot hybridization was performed using a Biorad dot-blot apparatus. Amplified DNA (~1 μg) was hybridized to normal and mutant ASOs. For the C117R mutation, the sequences of the normal and mutant ASOs were 5′-CTGGCCCTGTCAGCCC-3′ and 5′-CTGGCCCCGTCAGCCC-3′, respectively. For the L236P mutation, the sequences of the normal and mutant ASOs were 5′-TCTCTACCTTGCTCTCC-3′) and 5′-TCTCTACCCTGCTCTCC-3′, respectively. The underlined residues represent the mutated bases. Following hybridization at 37°C for at least 3 h, the blots were washed at room temperature for 30 min in 6 × SSC containing 0.1% SDS, and then were washed for 2 h in the same solution at either 48°C (C117R) or 51°C (L236P).

Results

Identification of ASB Exonic Point Mutations in MPS VI

As shown in table 1, each of the five unrelated MPS VI patients had residual ASB activity in cultured fibroblasts that ranged from ~0.4% to ~7% of the mean normal activity. To identify possible exonic mutations in the ASB gene that cause MPS VI, total fibroblast RNA was isolated from probands 1 and 2 and was reverse-transcribed into cDNAs. The ASB-coding sequences were then individually PCR amplified. Sequencing of the complete coding region from proband 1 revealed a single base substitution, a T-to-C transition in nt 349 of the full-length ASB cDNA, which predicted a cysteine-to-arginine substitution of residue 117 (C117R; fig. 1*A*). Of the 10 subclones sequenced, all contained the T-to-C transition, indicating the occurrence of a single homoallelic mutation in this family. In contrast, two different ASB exonic point mutations were identified in proband 2, who also was informative for the ASB polymorphism in nt 1072 (see below). A T-to-C transition in nt 707 of the ASB cDNA predicted a leucine-to-proline replacement at residue 236 (L236P; fig. 2*A*), and a G-to-A transition in nt 1214 predicted a cysteine-to-tyrosine substitution at residue 405 (C405Y; fig. 3). Of the 10 subclones sequenced from proband 2, 4 had the L236P mutation while 6 had the C405Y mutation.

Table 1

In Vitro ASA and ASB Levels in Cultured Fibroblasts from Normal Individuals and from Patients with MPS VI

Cell Line	Phenotype	ASB (nmol/h/mg)	ASA (nmol/h/mg)	ASB/ ASA	% Normal Mean ASA	% Normal Mean ASB
Normal individuals:		628	115			
MS-1	Normal			5.5	...	...
MS-2	Normal	754	162	4.7	...	...
MS-3	Normal	597	133	4.5	...	...
Mean		660	137	4.9		
MPS VI patients:						
MS-3[a]	Severe	12.4	223	.06	163	1.9
MS-4[b]	Mild	46.3	293	.16	213	7.0
MS-5	Severe	2.7	444	.006	324	.4
MS-6	Mild	39.8	153	.26	112	6.0
MS-7	Mild	39.1	365	.11	266	5.9

[a] Proband 1.
[b] Proband 2.

Confirmation of the C117R, L236P, and C405Y Mutations

To confirm the authenticities of the C117R and L236P mutations, dot-blot hybridization analyses were performed on PCR-amplified genomic DNAs from probands 1 and 2, from 3 other unrelated MPS VI patients, and from 60 normal individuals. As shown in figure 1*B*, dot-blot hybridization of genomic DNA from proband 1 confirmed that this MPS VI patient was homoallelic for the C117R mutation. Dot-blot hybridization analysis also confirmed that proband 2 was heteroallelic for the L236P mutation (fig. 2*B*). None of the three other unrelated MPS VI patients had either of these mutations, nor were they

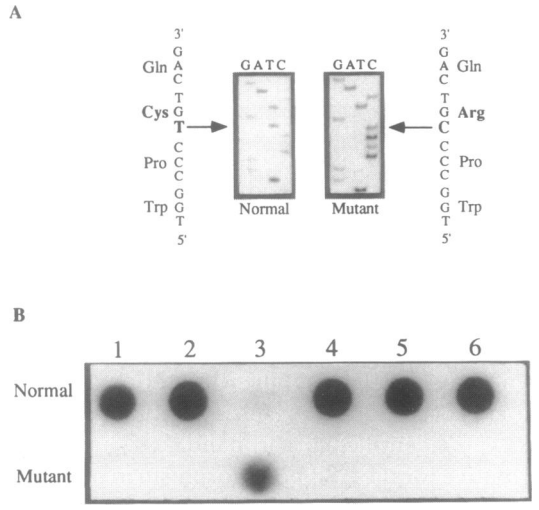

Figure 1 Identification of the C117R mutation in proband 1. *A*, Partial ASM cDNA sequence showing the T-to-C transition at nt 349 (arrow), which predicted a cysteine-to-arginine substitution at residue 117. *B*, Dot-blot hybridization with ASOs. Lanes 1–6 contained PCR-amplified genomic DNA from a normal individual, proband 2, proband 1, and MPS VI cell lines MS-5, MS-6, and MS-7, respectively.

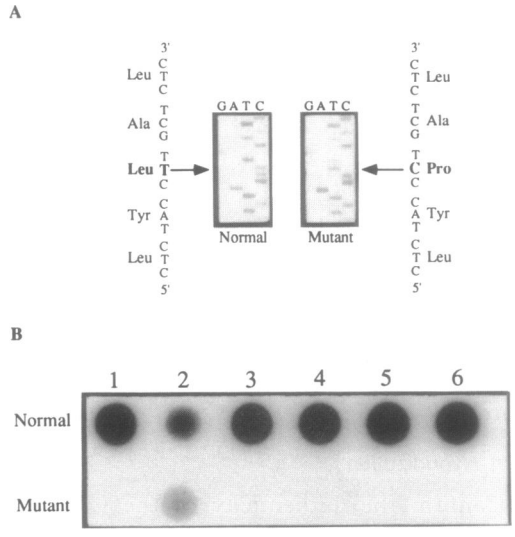

Figure 2 Identification of the L236P mutation in proband 2. *A*, Partial ASM cDNA sequence showing the T-to-C transition at nt 707 (arrow), which predicted a leucine-to-proline substitution at residue 236. *B*, Dot-blot hybridization with ASOs. Lanes 1–6 contained PCR-amplified genomic DNA from a normal individual, proband 2, proband 1, and MPS VI cell lines MS-5, MS-6, and MS-7, respectively.

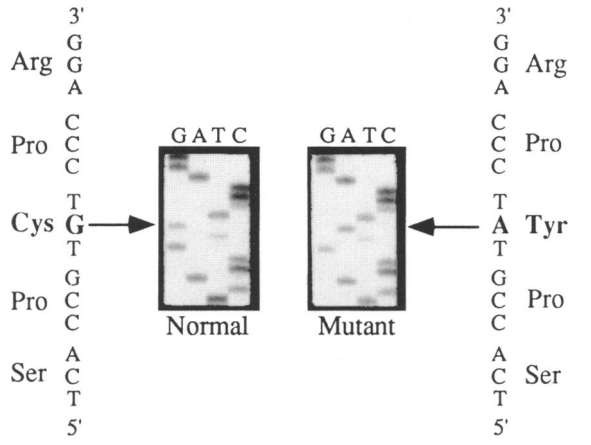

Figure 3 Identification of the C405Y mutation in proband 2. Partial ASM cDNA sequence showing the G-to-A transition at nt 1214 (arrow), which predicted a cysteine-to-tyrosine substitution at residue 405.

found in 120 normal ASB alleles, indicating that these lesions were not polymorphisms. Although genomic PCR amplification was not successful for the ASB region containing the C405Y mutation (presumably because of the presence of a large intron), the following results support the occurrence of these two ASB mutations in proband 2. First, 6 of 10 sequenced subclones from proband 2 contained the C405Y mutation, which is consistent with heteroallelism. Second, all six of these subclones had a polymorphic G at nt 1072 (Jin et al. 1991), whereas the four subclones containing the L236P mutation had an A in this position. Third, these point mutations and the polymorphism at nt 1072 were the only nucleotide changes found in the ASB-coding region from proband 2. Thus, these results indicated that both ASB alleles from proband 2 were sequenced, and they provided further evidence for heteroallelism of the L236P and C405Y mutations.

Discussion

In this communication, the first molecular lesions in the ASB gene that cause MPS VI have been described. The C117R mutation was identified in both mutant alleles from an MPS VI patient who had the severe form of the disease and about 2% of the mean normal ASB activity in cultured fibroblasts. It is unusual that a patient with a rare disease, such as MPS VI (i.e., occurring in fewer than 1;100,000 births), would be homoallelic for a mutation unless the mutant allele was common or the patient was a product of a consanguinous mating. The latter explanation is presumed to be likely for proband 1, since both of his parents were of English/Irish ancestry and may, in fact, have been related. The C117R mutation markedly alters the activity of ASB, perhaps by altering the active site and/or enzyme stability by means of replacement of a crucial cysteine residue by a positively charged arginine. In proband 2, who had a mild MPS VI phenotype and about 7% of the mean normal ASB activity in cultured fibroblasts, two mutant alleles were identified, L236P and C405Y. The occurrence of two different mutant alleles in this patient was supported by the fact that the common and rare forms of the exonic ASB polymorphism at nt 1072 segregated in all of the sequenced PCR products with either the L236P or the C405 mutation, respectively. One or both of these mutations may be responsible for the residual ASB activity and the milder phenotype of proband 2. Clearly, expression studies of the mutant alleles must be carried out to further investigate the effects of these amino acid alterations on the activity and stability of the ASB polypeptide.

Although the diagnosis of MPS VI is reliably made by demonstrating the markedly deficient ASB activity in isolated leukocytes or cultured cells, the accurate detection of heterozygotes has been difficult (McGovern et al. 1981). In addition, no reliable enzymatic tests have been developed to predict the severity of MPS VI in unrelated patients. Although the recent report of an immunologic assay for genotype/phenotype correlations is encouraging (Brooks et al. 1991), the panel of seven monoclonal antibodies required to perform these analyses is not generally available, and the tests themselves are labor intensive. Therefore, the identification of the specific molecular defects in MPS VI patients should facilitate more accurate carrier testing and provide the basis for genotype/phenotype correlations for this disease.

In summary, three first molecular lesions in the ASB gene which cause MPS VI have been described. Although it is premature to make genotype/phenotype correlations for this disease, these exonic point mutations provide insights into the nature of the molecular lesions causing MPS VI and the foundation for future investigations into the genetic heterogeneity underlying the mild, intermediate, and severe MPS VI phenotypes.

Acknowledgments

The authors thank Drs. Arthur Beaudet (Baylor College of Medicine) and Maurice Mahoney (Yale University School

of Medicine) for supplying clinical information on probands 1 and 2, respectively, and thank Sofiana Katz for expert technical assistance. This work was supported by a research grant (5 RO1 DK25759) from the National Institutes of Health and by a grant (5 MO1 RR00071) from the National Center of Research Resources, National Institutes of Health to the Mount Sinai General Clinical Research Center. C.E.J. was the recipient of a predoctoral fellowship (5 T32 HD-70105) in Mental Retardation and Developmental Disabilities from the National Institutes of Health.

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