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Molecular Genetic Defect Underlying α-L-Iduronidase Pseudodeficiency

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Summary

Mucopolysaccharidosis type I (i.e., Hurler, Hurler-Scheie, and Scheie syndromes) and type II (i.e., Hunter syndrome) are lysosomal storage disorders resulting from α-L-iduronidase (IDUA) deficiency and iduronate-2-sulfatase (IDS) deficiency, respectively. The a priori probability that both disorders would occur in a single individual is ~1 in 5 billion. Nevertheless, such a proband was referred for whom clinical findings (i.e., a male with characteristic facies, dysostosis multiplex, and mental retardation) and biochemical tests indicated these concomitant diagnoses. In repeated studies, leukocyte 4-methylumbelliferyl-α-L-iduronidase activities in this kindred were as follows: <1.0 nmol/mg protein/h in the proband and proband's clinically normal sister; 45.3 in mother; and 45.7 in father (normal range 65.0–140). Leukocyte L-O-(α-iduronate-2-sulfate)-(1→4)-D-O-2,5-anhydro[1-^{3}H]mannitol-6-sulfate activities were as follows: 0.0 U/mg protein/h in the proband; 5.7 in his sister; 4.9 in mother; and 15.0 in father (normal range 11.0–18.4). Multiple techniques, including automated sequencing of the entire IDS and IDUA coding regions, were employed to unravel the molecular genetic basis of these intriguing observations. The common IDS mutation R468W was identified in the proband, his mother, and his sister, thus explaining their biochemical phenotypes. Additionally, the proband, his sister, and his father were found to be heterozygous for a common IDUA mutation, W402X. Notably, a new IDUA mutation A300T was also identified in the proband, his sister, and his mother, accounting for reduced IDUA activity in these individuals; the asymptomatic sister, whose cells demonstrated normal glycosaminoglycan metabolism, is thus a compound heterozygote for W402X and the new allele. This A300T mutation is the first IDUA pseudodeficiency gene to be elucidated at the molecular level.

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Introduction

The mucopolysaccharidosis (MPS) disorders are a group of lysosomal storage diseases, each resulting from the deficiency of an enzyme required in the degradative pathways of sulfated glycosaminoglycans (GAG). Measured deficit of the appropriate enzyme activity has been considered a definitive and unequivocal method of diagnosis and defines each of the 10 known metabolic defects. Furthermore, quantification of the appropriate enzyme activity has been relied on for prenatal diagnosis and to assign the "carrier" genotype among relatives of an affected individual.

MPS type I is a spectrum of autosomal recessive conditions due to deficiency of α-L-iduronidase (IDUA, α-L-iduronide iduronohydrolase; E.C.3.2.1.76). Some 50 different mutations of the IDUA gene have been identified (Scott et al. 1992, 1993; Bach et al. 1993*b*; Clarke and Scott 1993; Moskowitz et al. 1993; Bunge et al. 1994, 1995; Clarke et al. 1994*b*; Tieu et al. 1995). The clinical phenotype is variable, ranging from Hurler syndrome to Scheie syndrome (Neufeld and Muenzer 1989; Gorlin et al. 1990; Hopwood and Morris 1990; Whitley 1993). The most severe presentation, Hurler syndrome, is diagnosed before age 2 years, affected children having characteristic facial features, hepatosplenomegaly, and kyphoscoliosis. Progression of the disorder results in corneal opacification, cardiac disease, skeletal abnormalities, and CNS damage. Older children are severely disabled, experience intellectual regression, and rarely survive to age 10 years. In contrast, individuals with Scheie syndrome are usually diagnosed in adulthood when progressive corneal clouding, carpal tunnel syndrome, and cardiac valve involvement become symptomatic. Such affected individuals have normal intellect and may have normal longevity. The third phenotype, Hurler-Scheie syndrome, is characterized by an intermediate age of diagnosis, variable skeletal and intellectual involvement, and premature demise.

In contrast, Hunter syndrome (MPS II) is an X-linked disorder resulting from defective iduronate-2-sulfatase (IDS) enzyme, the gene for which has been localized to Xq28 (Le Guern et al. 1990). Clinical phenotypes of severe Hunter syndrome overlap with those of Hurler syndrome. However, Hunter syndrome is usually differ-

entiated on the basis of its lack of corneal clouding and the absence of kyphoscoliosis.

Although Hurler syndrome and Hunter syndrome are recognized as "prototype" MPS diseases, they are relatively rare disorders, with incidence estimated as 1/50,000 and 1/100,000 births (Hopwood and Morris 1990; Lowry et al. 1990), respectively. Thus, the a priori probability that both disorders would occur in the same individual is ~1 in 5 billion. However, such a patient was referred to us for whom the clinical and biochemical findings indicated these concomitant diagnoses. Toward resolving such an unlikely occurrence, we extended our recent work with the powerful technique of automated DNA sequencing to find mutations of the IDS gene (Jonsson et al. 1995), and developed an analogous system for sequencing the entire IDUA coding region. This investigation led us, surprisingly, to identify the molecular genetic defect responsible for a pseudodeficiency allele of the IDUA gene.

Patients, Material, and Methods

Patients

The proband was first evaluated at 3½ years of age for gross motor delay, mild expressive language delay, and progressive changes in facial features and joints suggestive of an MPS storage disease. He was noted to be a "noisy breather" and had a history of frequent ear infections. Physical examination showed the proband to be 40 inches tall (50th percentile), to weigh 42.5 pounds (95th percentile), and to have a head circumference of 53 cm (>98th percentile). There was a prominent metopic suture and facial features were said to be "coarsened." The corneas were clear, the liver edge was palpated 2 cm below the right costal margin, and a grade II/VI systolic ejection murmur was appreciated. The back was straight, but other joints were limited in flexion and extension, owing to generalized contractures. Studies of urine included a positive Berry spot test and abnormally high excretion of dermatan sulfate and heparan sulfate. Repeated measurements of leukocyte IDUA and plasma IDS activities demonstrated complete deficiency of both enzymes, levels diagnostic of Hurler syndrome and Hunter syndrome, respectively.

The proband's parents denied consanguinity and had a normal 6-year-old daughter. In addition, a maternal uncle was reported to have died at age 11 having "coarse facial features" and mental retardation; however, no diagnostic studies had been done, and no tissue has been available for diagnostic testing.

Examination of the proband at this center at 4½ years of age confirmed the reported physical findings. Extensive histocompatibility typing of all family members confirmed the relationships and did not suggest consanguinity. Additional diagnostic studies were undertaken as described below. Informed consent was obtained for molecular genetic studies according to the policies of the institutional review board at the University of Minnesota. At the time of this writing, the proband is 11 years of age and has been reported to show progressive intellectual deterioration, while his 14-year-old sister continues to be normal in all respects.

Determination of Enzyme Activities

Leukocytes were prepared by differential sedimentation on dextran followed by two cycles of hypotonic hemolysis (Lichtman 1990). For some individuals, a lymphoblastoid cell line (LCL) was prepared by transformation with Epstein-Barr virus.

The activity of IDUA was measured using fluorogenic substrate 4-methylumbelliferone (MU) α-L-iduronide (Calbiochem) and expressed as nmol MU/mg protein/h, or nmol MU/ml plasma/h, as described elsewhere (Whitley et al. 1987). It is notable that the assay was originally developed to optimize human leukocyte IDUA activity at 37°C with respect to reaction pH (3.3) and substrate concentration (2.85 mM, >10-fold above the K_m). Under these conditions, the reaction was found to be linear with respect to reaction time for ≥3 h, although reactions were either 30 min or 2 h in the current study. Protein concentration was measured with Coomasie blue (Bio-Rad) by the method of Bradford (1976).

Plasma IDS enzyme activity was measured by Minnie Deanching in the laboratory of Dr. George Hoganson (University of Illinois at Chicago) according to a previously published method (Wasteson and Neufeld 1982; Clarke et al. 1990). One unit of enzyme activity was defined as the percent of ^3H substrate converted to product. Specific enzymatic activity was reported as U/mg protein/h.

The activities of N-acetyl-α-glucosaminidase (Marsh and Fensom 1985) and β-glucuronidase (Hall et al. 1973) were measured in plasma using the appropriate MU-glycoside substrates (Sigma). Activities were expressed as nmol MU/ml plasma/h.

$^{35}SO_4$-GAG Labeling and Assay

For studies assessing GAG metabolism, LCL cells were grown for 2 d in minimal essential medium (MEM, 410-2300, Gibco/BRL, 1.6 g/liter $NaHCO_3$ at pH 6.9) with 140 mg/liter $CaCl_2$, 15% fetal bovine serum (Hyclone) and 2 mM glutamine. Cellular GAG was metabolically labeled by addition of $^{35}SO_4$ (4 μCi/ml, Amersham) to culture media. After cultivation for an additional 2 d, LCL were washed twice with PBS, lysed with 0.1 ml of 0.5 M NaOH, and then neutralized with 0.05 ml 1 M HCl. Macromolecular material was separated from unincorporated $^{35}SO_4$ by centrifugal column chromatography (Bio-Gel P10, Bio-Rad). Incorporated ra-

Table 1

Oligonucleotides for IDUA Gene Amplification and Sequencing

Primer	Orientation[a]	Sequence[b]	Location[c]
IDUA1	+	CAGTGCAGCCCGAAGCCCCGCAGT	47→70[d]
IDUA2	−	CGCTGACTGCCGGTGGAGGTGCAA	2096→2073[d]
IDUA3	+	AGGCGGGCGTGCGGCTGGACTACA	840→863[d]
IDUA4	−	GCGGGTGTCGTCGCTCGCGTAGAT	1429→1406[d]
IDUA5	+	GGCCCCGCACCTGGTGCAGG	169→188
IDUA6	−	CTCCTCCAGAAGCGCCGCAGGG	234→213
IDUA7	+	TGGCTGCTGGAGCTTGTCAC	362→381
IDUA8	−	TCCCTGAGAAGGTCCAAGTACC	456→435
IDUA9	+	CGCATGTTTCCAAGTGGAAC	597→616
IDUA10	−	CATGGAGACGTTGTCAAAGTCG	667→646
IDUA11	+	GCATCAGAACCTGCTACTGG	1072→1091
IDUA12	−	CTCCAGGATGGAGATGGAGCTG	910→889
IDUA13	+	TCCTGGACAGCAACCACACG	1320→1339
IDUA14	−	GGTGTTGTTGACCTGGAAGC	1210→1191
IDUA15	+	CGTCTTCCCCACGGCAGAGC	1567→1586
IDUA16	−	CCGGAACTGCTCTGCCGTGG	1594→1575
IDUA17	+	GGTTCTGGTCTGGTCGGATG	1780→1799
IDUA18	−	CTGAGAGAACTGGATCTCG	1849→1830
ID SIN2	+	CACCTTGCAGGCTCCCACATGC	−102 from exon 3
ID ASIN4	−	CCACCAATGTATCTCCTGACCA	+105 from exon 4
IDUA20	−	GGTcGAcGTGCAACCCACTGGG[d]	2085→2064
IDUA21	+	AGCCCGAAttCCCGCAGTCC	53→72

[a] A plus sign (+) refers to sense strand, and a minus sign (−) indicates antisense strand.

[b] Lowercase letters represent changes from normal sequence included for genetic engineering purposes.

[c] For convenience, enumerated according to the initial published sequence (Scott et al. 1991). Subtract 88 nt to identify position with respect to the first nucleotide of the ATG initiation codon.

[d] Bach et al. (1993*b*).

diosulfate then was quantified by scintillation counting in 5 ml liquid fluor (Ecolume, ICN Biomedicals).

Oligonucleotide Primers

Oligonucleotides used in this study for amplification and sequencing of IDUA are listed in table 1. Those for amplification and sequencing of IDS are described elsewhere (Jonsson et al. 1995). Primers were synthesized on an Applied Biosystems 391 DNA synthesizer.

Generation of IDUA cDNA

Total cellular RNA was isolated from leukocytes (obtained from 10 ml blood) using TRIzol reagent (Gibco/BRL) as described by the manufacturer. The IDUA cDNA was generated from mRNA in a 20-μl reaction containing Moloney murine leukemia reverse transcriptase (200 U, Gibco/BRL), 1 mM of each dNTP, 15 U RNasin (Promega), 10 mM DTT, and 50 pmol of antisense primer IDUA2 (table 1). Temperature during reverse transcription (RT) was 37°C for 30 min, followed by a linear gradient from 37°C to 55°C over 15 min. The reaction was stopped by heating at 94°C for 6 min.

The cDNA preparation was used as template for PCR to amplify the entire IDUA coding region. An aliquot of cDNA template was added to a mixture containing 50 pmol primer IDUA1 (table 1), buffer (1 × optimized buffer A kit, Invitrogen), 5% dimethyl sulfoxide (DMSO) and 5% glycerol. The mixture was heated (denaturation) at 96°C for 8 min, after which 2.5 U *Taq* DNA polymerase (Boehringer-Mannheim) was added for amplification: 35 cycles of 97°C for 40 s, 63°C for 30 s, 72°C for 2 min 40 s, and a final extension of 5 min at 75°C. Aliquots (5 μl) of the primary PCR reaction were reamplified in two separate 100-μl reactions (using 5 U *Taq* polymerase, 1 × optimized buffer A, 5% DMSO, 5% glycerol) with nested primers IDUA21 and IDUA4, and IDUA3 and IDUA20, to generate 5′-and 3′-end secondary amplicons, respectively. After denaturation at 96°C for 5 min, *Taq* polymerase was added, followed by 30 cycles of amplification: 96°C for 40 s, 63°C for 30 s, and 72°C for 2 min, with a final extension of 5 min at 72°C.

Secondary IDUA amplicons were analyzed by electrophoresis of reaction aliquots (8 μl) in 0.7% agarose gels containing ethidium bromide (0.5 μg/ml buffer) followed by visualization of products under UV light. The concentration of DNA product was estimated by comparison to a standard λ/*Hin*dIII marker (Gibco/BRL).

Isolation and Amplification of IDUA Genomic DNA

Genomic DNA was isolated from peripheral blood or LCL cells (Puregene DNA isolation kit, Gentra Systems). PCR amplification was carried out using 1–2 μg of genomic DNA as template and the same conditions as for secondary PCR of cDNA, except that 40 cycles were used. Aliquots (8 μl) of reactions were analyzed by agarose gel electrophoresis as described above.

Sequencing Techniques

For sequencing, PCR products were washed twice in 2 ml deionized-distilled water using Centricon-100 centrifugal concentrators (Amicon). DNA template (50–100 ng) was then copied and labeled using the PRISM™ Ready Reaction Dye Deoxy™ Terminator cycle sequencing kit (Applied Biosystems) as recommended by the manufacturer, i.e., 25 cycles of 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min. Automated sequencing was done as described elsewhere (Jonsson et al. 1995).

Restriction Endonuclease Analysis

To confirm the R468W mutation of the IDS gene, genomic DNA was amplified using primers IDS7 and IDS20 to generate a 441-bp product. The amplicon was purified by Bio-Gel P-10 (Bio-Rad) size exclusion centrifugal column chromatography from which 10 ml aliquot was used for restriction digestion in a 20-ml reaction mixture containing 10 U *Msp*I and 1 × NEB buffer 2 (New England Biolabs) incubated 1 h at 37°C. Products were separated by electrophoresis in 2% agarose gel stained with ethidium bromide and visualized under UV light.

For confirmation of the IDUA mutation W402X, the primer pair IDUA11-IDUA16 was used to generate an 857-bp DNA fragment (from genomic template) or 524-bp cDNA fragment. After purification (as described above), 5 μl of the PCR product was digested with 5 U *Mae*I (Boerhinger-Mannheim) in a 20-μl reaction containing 1 × buffer recommended by the manufacturer. After incubation for 2 h at 45°C the reaction products were separated in 1.7% agarose gel and visualized as described above.

Results

Deficient IDUA and IDS Enzyme Activities

To confirm reports indicating the unexpected dual enzyme deficiency in this patient, leukocyte IDUA and plasma IDS enzyme activities were determined for the proband and family members (table 2). IDS enzyme activity was undetectable in the proband (0.0 U/mg/h), normal in the father (15.0), and at half-normal levels in the proband's mother (4.9) and sister (5.7). These data supported the diagnosis of Hunter syndrome in the proband and of heterozygote status of the mother and sister.

Measurement of leukocyte IDUA activity revealed a profound deficiency in the proband (table 2). Leukocyte IDUA activity was absent (<1.0 nmol/mg/h) by the usual assay method involving prior cryolysis of cells and also undetectable in unfrozen cells disrupted by sonication. Activity levels of the mother (45.3) and father (45.7) were half-normal and characteristic of carriers for MPS I (heterozygote range 65–140). Remarkably, the apparently normal sister of the proband was found to have no detectable IDUA activity (<1.0).

Lysosomal enzyme activities were also measured in plasma (table 2). Only the proband (but not his sister) had somewhat high levels of N-acetyl-α-glucosaminidase and β-glucuronidase. These levels were consistent with the slight elevations observed as a secondary abnormality in many lysosomal diseases yet were not the extremely high levels observed in the defects of lysosomal protein maturation and trafficking, i.e., mucolipidosis types II and III.

$^{35}SO_4$-GAG Accumulation in LCL

Next, we compared $^{35}SO_4$-GAG accumulation by LCL derived from family members (fig. 1). The proband's cells exhibited continued $^{35}SO_4$-GAG accumulation, a pattern pathognomonic for an MPS defect. In contrast, no $^{35}SO_4$-GAG accumulation was observed in cells from the sister and father.

Identification of IDS Mutations

Sequencing of the entire IDS coding region in the proband revealed a missense mutation, 1402 C→T, as the sole abnormality (fig. 2). This mutation results in the substitution of tryptophan for arginine at position 468 of the IDS protein (i.e., R468W) and is the most common mutation in patients with Hunter syndrome (Whitley et al. 1994). For the sister and mother, the region of cDNA spanning codon R468 was sequenced in both directions by using primers IDS7 and IDS20. The same base substitution, in heterozygosity with the normal nucleotide, was observed. Sequencing results were confirmed by *Msp*I restriction endonuclease analysis (fig. 3). The finding of R468W accounted for absent IDS enzyme assay in the proband and for heterozygote levels in his sister and mother.

Identification of IDUA Mutations

To identify mutations of the IDUA gene, we developed a method for automated sequencing of the entire IDUA coding region. This method of sequencing RT-PCR products derived from patient cells (e.g., fresh leukocytes or cultured LCL), is analogous to the terminator-labeled method we developed for identification of IDS mutations causing Hunter syndrome (Jonsson et al. 1995). As illustrated in the current study (see below), the method is satisfactory despite the challenges inherent

Table 2

Enzyme Activities in the Kindred Exhibiting Concomitant Deficiency of α-L-Iduronidase and Iduronate-2-Sulfatase

	α-L-Iduronidase, Leukocytes[a]	α-L-Iduronidase, Plasma[b]	Iduronate-2 Sulfatase, Plasma[c]	N-acetyl-α-Glucosaminidase, Plasma[b]	β-Glucuronidase, Plasma[b]
Proband	<1.0	<.20	.0	47.5	111
Sister	<1.0	<.20	5.7	24.2	32.6
Mother	45.3	5.10	4.9	18.6	33.4
Father	45.7	6.56	15.0	30.0	46.6
Normal homozygote	65.0–140	6.87	11.0–18.4	17.1; 20.5	19.0; 27.1
Heterozygote	17–70		5.5		

[a] Leukocyte IDUA activity expressed as nmol MU/mg protein/h.
[b] Plasma enzyme activities expressed as nmol MU/ml plasma/h.
[c] Plasma IDS enzyme activity expressed as U/mg protein/h.

to simultaneous analysis of both alleles. Accuracy is dependent on visual comparison of sequencer output tracings and must go beyond simple acceptance of the computer "calls" of the most likely nucleotide. In some cases where there is ambiguity, a definitive evaluation requires the analysis of multiple sequencing reactions that use different primers, preferably reading in opposite directions, i.e., the sense strand and antisense strand.

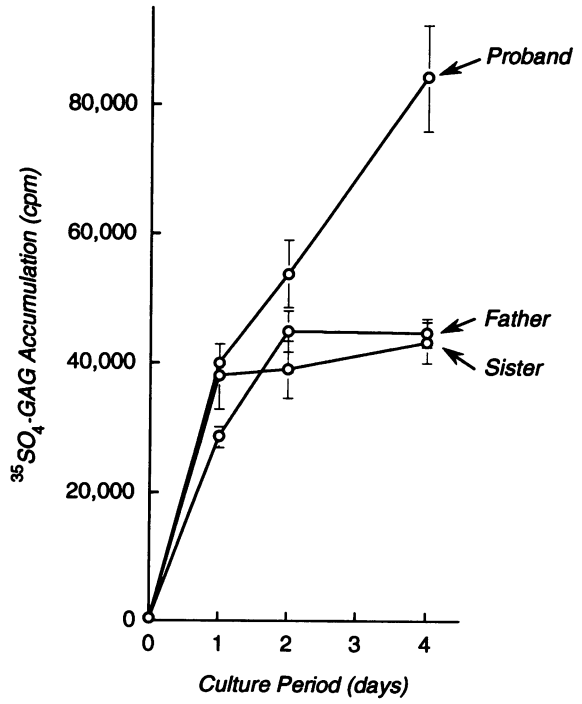

Figure 1 $^{35}SO_4$-GAG accumulation over time. In LCL from the proband, $^{35}SO_4$-GAG accumulation was progressive over 4 d in culture, characteristic of defective catabolism in MPS diseases. In contrast, the proband's sister and father exhibited normal $^{35}SO_4$-GAG metabolism. Each point is the mean of triplicate cultures (bars represent ±1 SD).

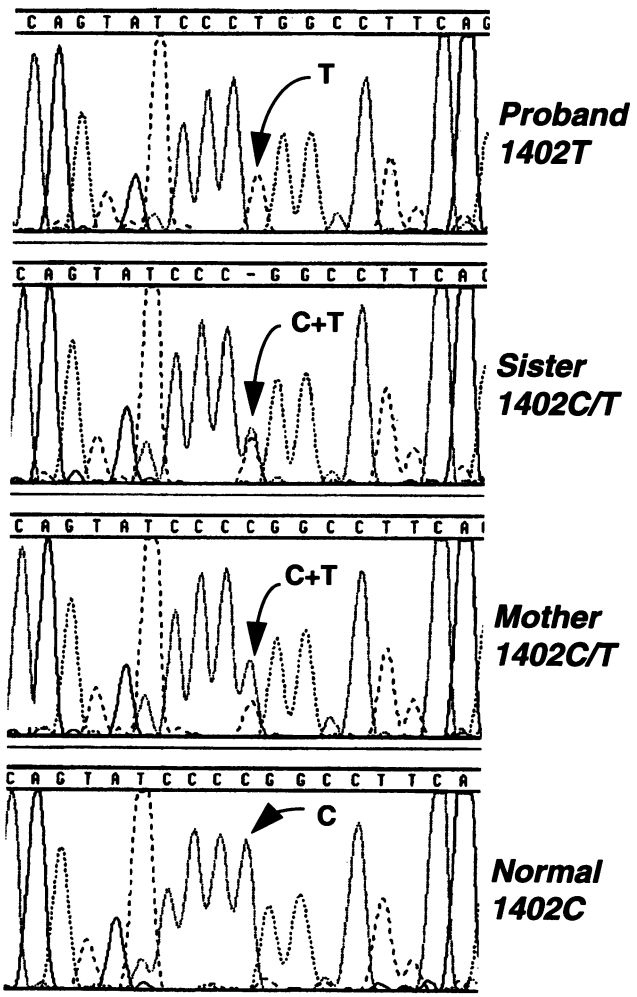

Figure 2 Automated sequencing of IDS cDNA (generated by direct RT-PCR amplification of leukocyte mRNA). Sequencing identified the mutation 1402C→T in the proband as the only change in the entire coding sequence. The same method indicated that the proband's mother and sister were heterozygotes for this mutation.

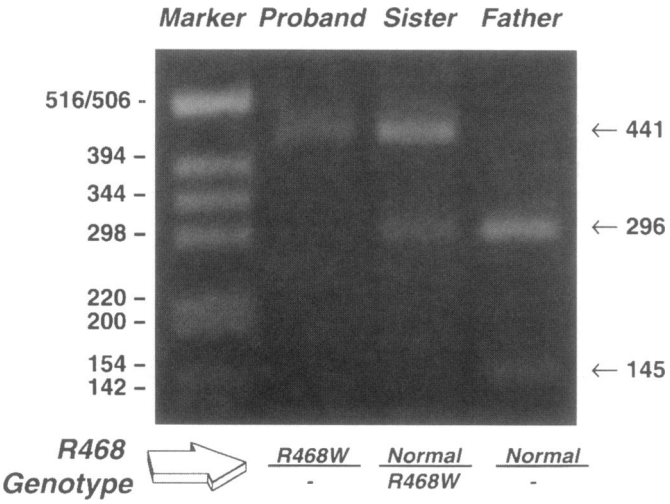

Figure 3 R468W mutation, confirmed by restriction endonuclease digestion with *Msp*I in the IDS PCR products amplified from genomic DNA. Several different mutations abolish the diagnostic *Msp*I restriction site. For alleles that have mutations at this site, such as R468W, the 441-bp fragment remains uncut, as in the proband. The presence of three bands (i.e., 441, 296, and 145 bp) indicates a heterozygote genotype with both alleles present, as in the case of the sister. If only the normal allele is present, the 441-bp PCR product is cleaved into 296-bp and 135-bp fragments, as in the case of the proband's father.

Initially, the entire IDUA coding regions for the proband and his sister were sequenced using cDNA as a template. The sequencer tracings of both sibs revealed a single, mutant A peak in place of the normal nucleotide 898G in exon 7 (fig. 4*a* and *b*). This mutation is predicted to change the usual alanine at amino acid 300 to threonine, i.e., A300T. Sequencing of IDUA cDNA from the proband's mother revealed two simultaneous peaks corresponding to G and A at the same position in the mother (fig. 4*e*); thus, she appeared to be heterozygous for this mutation. However, no mutations changing an amino acid were found anywhere in the entire cDNA sequence of the proband's father, and he appeared to have only the normal G at nt 898 (fig. 4*f*). In sum, these results were ambiguous with respect to whether the proband was homozygous, or possibly heterozygous, for the A300T mutation.

To further clarify these observations, genomic DNA from each family member was amplified using the primer pair IDUA3-IDUA14 (amplifying all of exon 7), and the PCR products were sequenced using primer IDUA3. In contrast to the results obtained for cDNA, the proband and his sister clearly showed two overlapping peaks, G and A, at cDNA position 898 in genomic DNA (fig. 4*c* and *d*). Analysis of paternal genomic DNA showed no mutation at this site (data not shown).

The observation that some mutations may result in unequal expression of alleles in autosomal recessive con-

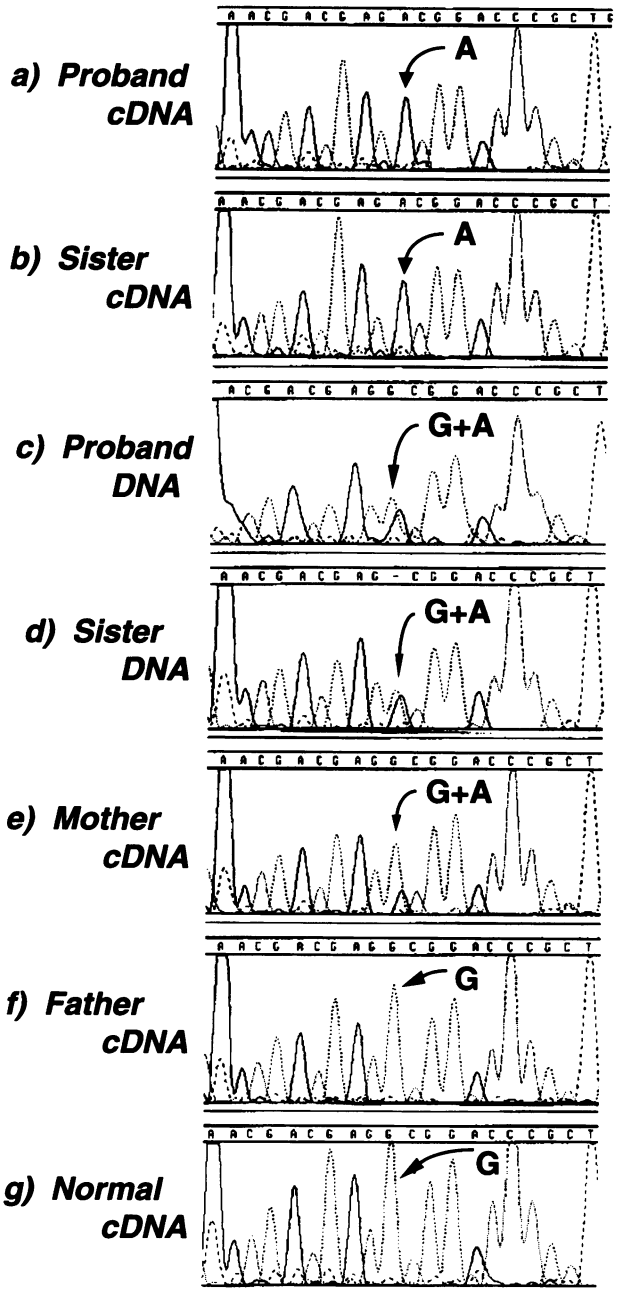

Figure 4 Identification of the A300T mutation. A system for automated sequencing of the IDUA coding region was developed that identified the A300T mutation but yielded somewhat conflicting results in cDNA when compared with genomic DNA. Sequence tracings of RT-PCR products (cDNA) from the proband and his sister showed a single, mutant A peak in place of the normal nt 898G (i.e., mutation 898G→A, panels *a* and *b*). In contrast to the tracings for cDNA, analysis of genomic DNA showed the proband and his sister to have two overlapping peaks, G and A, at this position (panels *c* and *d*), indicating that both individuals are heterozygous for this mutation. Sequencing of cDNA from the proband's mother revealed two simultaneous peaks corresponding to G and A (panel *e*), indicating that she is heterozygous for this mutation. No mutations were found in the entire cDNA sequence of the proband's father, and he appeared to have only the normal G at nt 898 (panel *f*).

ditions has been well documented (Urlaub et al. 1989; Bach et al. 1993; Dietz et al. 1993; Hoffmeyer et al. 1994; Zhang et al. 1994). This phenomenon would explain the different sequence results obtained from cDNA and genomic DNA for the proband and his sister. Thus, we anticipated a second IDUA mutation in this family and undertook sequencing of the entire IDUA coding region by using genomic DNA for reaction template. The following primer pairs were used to generate PCR fragments for analysis: IDSIN2-IDUA10, IDUA9-IDUA12, IDUA3-IDUA14, IDUA 11-IDUA16, IDUA15-IDUA18, and IDUA17-IDUA20, spanning exons 3–14 and intron-exon borders (table 1). Both external and nested primers were used for sequencing of the amplicons, where possible, in both directions. In this fashion, the proband was found to have the common mutation 1206C→T, i.e., W402X, in heterozygosity with the normal allele (fig. 5).

Heterozygosity of the proband for W402X was confirmed by restriction digestion with *Mae*I (Scott et al. 1993) of a genomic DNA PCR product IDUA11-IDUA16. As shown in figure 6, this method also indicated that both the proband and his sister were carriers of the W402X allele that was transmitted from the father. However, *Mae*I did not cleave the respective 524-bp cDNA PCR products generated with the same primer pair (data not shown). This latter finding was in accord with the results of cDNA sequencing and suggested preferential amplification of the allele that did not contain the W402X mutation; that is, messenger RNA for the W402X allele was relatively decreased, presumably because of decreased stability.

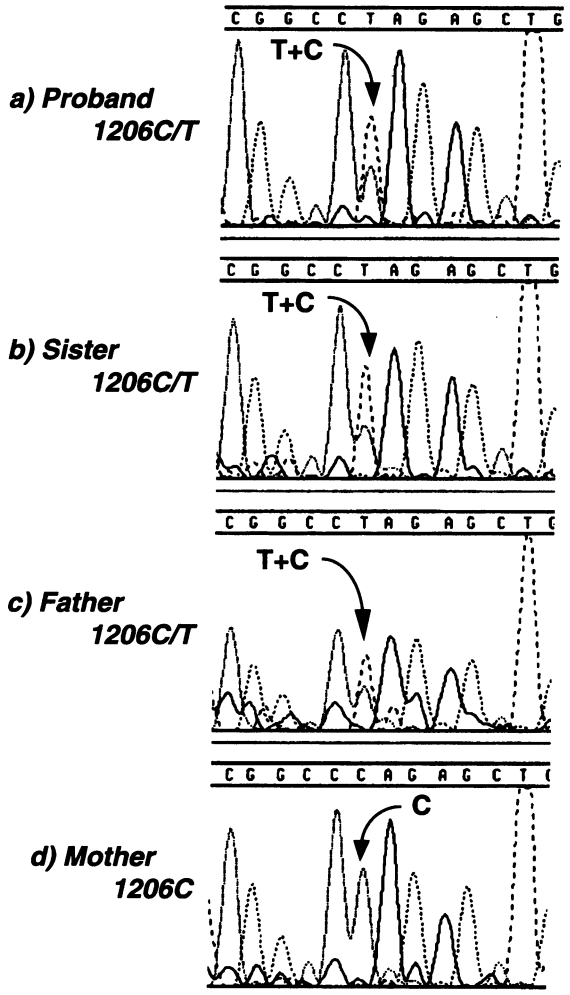

Figure 5 Automated sequencing of genomic DNA, identifying the 1206G→A mutation of the IDUA gene, i.e., W402X, in the proband (panel *a*), his sister (panel *b*), and his father (panel *c*). Sequencing in the antisense direction reveals the mutation 1206C→T in the antisense strand shown here. The normal nucleotide was observed in the mother's sequence (panel *d*).

Polymorphisms

Concomitant with identification of A300T and W402X mutations of the IDUA gene, three polymorphisms were identified in the proband and his sister, thus designating an IDUA haplotype presumably transmitted from the father: L118, observed in other patients (Scott et al. 1993); 1082c→t of intron 4; and 3976c→a of intron 13, numbered according to the published sequence (Scott et al. 1992), previously undescribed.

Transmission of a Pseudodeficiency Allele

In summary, transmission of three different mutations is responsible for the remarkable observations in this family (fig. 7). The common IDS mutation R468W was transmitted to the proband and his sister, from maternal relatives. This appears to be the cause of the undiagnosed MPS phenotype in the proband's uncle. The most common IDUA mutation associated with Hurler syndrome, W402X, was transmitted from the father to the proband and the proband's sister, reducing IDUA enzyme activity by half in each of these individuals. A previously unreported IDUA mutation A300T was transmitted from the mother to the proband and his sister, which, in compound heterozygosity with the W402X mutation, resulted in the absence of measurable IDUA activity in both children. Because the proband's sister was entirely unaffected and her cells were found to have normal GAG metabolism, the W402X/A300T compound heterozygote genotype must be considered an IDUA pseudodeficiency state.

Discussion

Evaluation of This Unique Family

Clinical features and family history suggested that the proband in this family was likely to have Hunter syndrome. The absence of corneal clouding and minimal

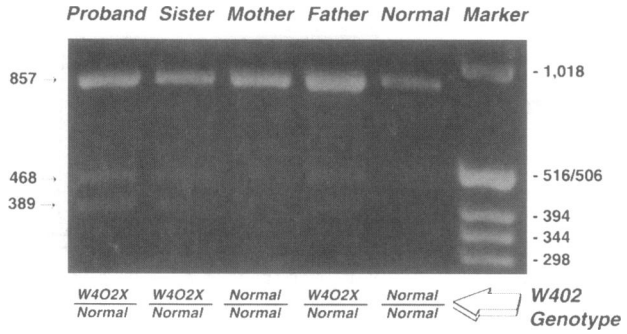

Figure 6 Restriction endonuclease digestion used to confirm the W402X mutation of IDUA in PCR-amplified products amplified from genomic DNA. The W402X mutation creates a *Mae*I restriction site. Electrophoresis in 1.7% agarose gel revealed three bands, 857 bp (uncleaved) and two smaller bands (468 bp and 389 bp), in the proband and his sister, confirming that each was heterozygous for W402X and a normal sequence. The mother showed only one 857-bp band, indicating that she did not carry the mutation, while the father showed three bands, consistent with heterozygosity for W402X.

nature of the kyphoscoliosis often distinguish patients with severe Hunter syndrome from those children with Hurler syndrome. The diagnosis of Hunter syndrome was further supported by the fact that a maternal uncle had died at age 11 with an MPS phenotype and a family history that would be consistent with transmission of a single X-linked mutation. However, the concurrent absence of IDUA enzyme deficiency was confirmed by our tests and merited further study.

Our subsequent investigations of the family determined that the unaffected sister of the proband also had no detectable IDUA activity. Other studies indicated that the proband's sister had an otherwise normal clinical and biochemical phenotype, including normal urinary GAG excretion and normal levels of other lysosomal enzymes. Plasma N-acetyl-α-glucosaminidase and β-glucuronidase were not markedly elevated as in the defects of lysosomal enzyme trafficking (i.e., mucolipidosis types II and III), nor were they slightly elevated, as is commonly observed in other lysosomal diseases (as illustrated by the proband). Further, cultured LCL cells from the proband's sister did not show pathologic $^{35}SO_4$-GAG accumulation. In this clinical context, deficiency of MU-α-L-iduronidase suggested the presence of one or more IDUA pseudodeficiency alleles.

Molecular studies were undertaken to further elucidate the molecular genetic basis of these findings. Sequencing of the IDS gene identified R468W, the most common mutation causing the severe form of Hunter syndrome (Whitley et al. 1994). The same mutation was found in the proband's sister and mother. Such observations (Crotty et al. 1992; Hopwood et al. 1993; Jonsson et al. 1995) are leading to the conclusion that codon 468 of IDS is a mutational hotspot.

To characterize the presumed IDUA mutations, we developed a method for automated sequencing of the entire IDUA coding region. Analogous to our recently developed method for automated sequencing of IDS cDNA, this method appears to identify all mutations of the coding region, the vast majority of mutations causing MPS I. In this family, sequencing revealed two IDUA mutations in the coding region, mutations that were present in both the proband and his sister. One mutation, W402X, is the most common change associated with Hurler syndrome, causing premature termination in exon 9. As observed by others (Bach et al. 1993*b*; Clarke et al. 1994*b*), mRNA from the W402X allele was markedly reduced; the W402X allele was not detected either by sequencing or by restriction digestion of the RT-PCR product. The mutation was detected only in analysis of genomic DNA.

The second IDUA mutation was A300T. No other mutations leading to an amino acid change or abnormal intron-exon borders (i.e., products of alternative splicing) were found. Although several dozen IDUA mutations and polymorphisms have been identified thus far, the A300T mutation has not been previously observed. The significance of a change at this amino acid is further suggested by the fact that A300 is conserved across spe-

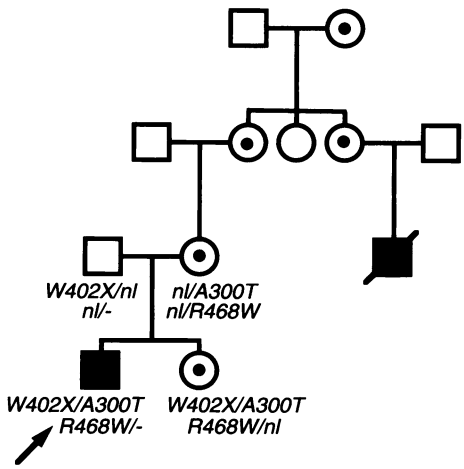

Figure 7 Family pedigree illustrating transmission of the mutant alleles identified in this family, including IDUA (upper genotype) and IDS alleles (lower genotype) as well as the corresponding normal alleles (nl). The proband (*arrow*) was determined to be a compound heterozygote for the most common mutation causing Hurler syndrome (W402X) and the newly characterized IDUA pseudodeficiency allele (A300T). The proband is also hemizygous for the most common allele associated with Hunter syndrome (R468W). The proband's sister has the same IDUA and IDS genotypes as the proband, except that the sister has an additional normal IDS allele. The fact that she has no features of MPS disease established the A300T allele as a benign, pseudodeficiency mutation. Several female relatives are presumed to be carriers for Hunter syndrome (represented by ⊙), on the basis of the observation that the proband's uncle died at age 11 with an undiagnosed MPS phenotype.

Table 3

Residual Methylumbelliferyl-α-L-Iduronidase Enzyme Activity Due to a Pseudodeficiency Allele in Six Compound Heterozygotes for a Pseudodeficiency Allele and an Allele for Hurler Syndrome

KINDRED (reference)	SUBJECT	SUBJECT GENOTYPE (pseudodeficiency/other allele)	MU-α-L-IDURONIDASE ACTIVITY[a]	
			Leukocytes	Cultured Cells
1 (Whitley et al. 1987)	Index case	Pseudodeficiency/MPS I H	3	7[b]
2 (Gatti et al. (1985)	Mother	Pseudodeficiency/MPS I H	15.3	14.5[b]
	Fetus	Pseudodeficiency/MPS I H	...	15–25[c]
3 (Taylor and Thomas 1993)	"Normal control"	Pseudodeficiency/?	5	10[b]
4 (Current report)	Proband	A300T/W402X	<2	...
	Sister	A300T/W402X	<2	...

[a] Methylumbelliferyl-α-L-iduronidase enzyme activity in subjects who are proved, or presumed, to be compound heterozygotes for a pseudodeficiency allele and a proved, or suspected, allele for Hurler syndrome. Enzyme activity in leukocytes or cultured cells is expressed as a percent of the mean of a normal population.

[b] Cultured skin fibroblasts.

[c] Cultured amniocytes.

cies among the known homologues, including the human (Scott et al. 1991), canine (Stoltzfus et al. 1992), murine (Clarke et al. 1994*a*), and leporine (E. L. Aronovich and C. B. Whitley, unpublished data) IDUA genes. It would appear that A300T alters catalytic activity toward the artificial MU α-L-iduronide substrate but does not have an effect on the clinical phenotype. As demonstrated in this family, the A300T mutation causes half-normal levels of IDUA activity when coexistent with the normal allele, e.g., in the proband's mother. It results in complete absence of enzyme activity when found in a compound heterozygote for a pathologic IDUA allele, e.g., the proband's sister.

α-L-iduronidase Pseudodeficiency Alleles

Clinical observations have long suggested the existence of multiple IDUA alleles causing at least three disease phenotypes, a historic nosology currently being confirmed by molecular genetic studies. One group of mutations causes the most severe phenotype, Hurler syndrome; five common mutations have been identified (Scott et al. 1993; Clarke et al. 1994*b*) as well as several other more rare mutations (Bach et al. 1993*b*; Moskowitz et al. 1993; Scott et al. 1993; Bunge et al. 1994; Clarke et al. 1994). Different mutations have been associated with the mild phenotype of Scheie syndrome (Scott et al. 1993), and others have been associated with the intermediate phenotype, Hurler-Scheie syndrome (Bunge et al. 1995; Tieu et al. 1995; E. L. Aronovich and C. B. Whitley, unpublished results). A fourth category of mutations must be considered, those that result in the apparent absence of IDUA enzyme activity by in vitro assays but that are not associated with a pathologic phenotype, i.e., the IDUA pseudodeficiency alleles.

The clinical settings in which such pseudodeficiency states have been discovered illustrate the significance of such alleles for which there is ongoing concern, especially for prenatal diagnosis. Only three reports of IDUA pseudodeficiency have been published, this sibship becoming the fourth. The first case, presented by us, was the mother of a child who had classic Hurler syndrome. Two subsequent pregnancies were terminated on the basis of low IDUA activity. On later evaluation of this family by us, the mother was found to have only 3%–7% of normal IDUA levels in leukocytes, plasma, tears, and cultured skin fibroblasts. When first presented (Whitley 1986), lack of previous reports of IDUA pseudodeficiency made this initial report quite controversial, and the IDUA pseudodeficiency state was not generally accepted until kinetic studies (Whitley et al. 1987) and another kindred transmitting relatively low IDUA activity (Gatti et al. 1985) were published. In this latter instance, a woman had given birth to a child with Hurler syndrome and was herself found to have unusually low IDUA enzyme activity. This became a significant clinical concern when amniocytes from her subsequent pregnancy were also found to have very low IDUA enzyme activity. The finding of normal $^{35}SO_4$-GAG metabolism argued that the fetus would be unaffected. After delivery, the child was determined to be normal and did not have Hurler syndrome. A third case was identified in a "normal control" specimen obtained in parallel with a diagnostic test (Taylor and Thomas 1993). Thus, the existence of IDUA pseudodeficiency alleles has become more widely accepted (Thomas 1994), although none of these cases have been characterized at the molecular level. The current report presents a fourth example recognized during the evaluation of a child with an apparent MPS storage disease. All but one example of IDUA pseudodeficiency have been discovered in an individual

proved to be heterozygous for Hurler syndrome. The exceptional case (Taylor and Thomas 1993) may have been such a compound heterozygote also. Lack of experience with such cases implies that pseudodeficiency mutations are relatively rare; however, there is no estimate of their actual frequency. Nevertheless, the consequences of pseudodeficiency genes are significant, particularly for prenatal diagnosis.

Previous reports provide sufficient information for a preliminary comparison and speculation regarding the molecular heterogeneity of alleles on the basis of measured IDUA activity (table 3). It is notable that the first case we reported had low, but detectable, levels of IDUA enzyme activity (2.9–4.0 nmol/mg/h) when measured in the same assay that yielded undetectable levels (<1 nmol/mg/h) in the proband and sister of the current report. Other cases appear to have relatively higher IDUA activity. Although differences in assay conditions (i.e., substrate concentration, pH, method of cell disruption, etc.) could also account for variances in measured activity between kindreds, we speculate that such differences reflect intrinsic molecular heterogeneity and that at least one additional pseudodeficiency mutation, and possibly several, will be identified.

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