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Mutational Analysis of a Patient with Mucopolysaccharidosis Type VII, and Identification of Pseudogenes

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Summary

PCR of cDNA produced from patient fibroblasts allowed us to determine the paternal mutation in the first patient reported with β-glucuronidase-deficiency mucopolysaccharidosis type VII (MPS VII). The G→T transversion 1,881 bp downstream of the ATG translation initiation codon destroys an *Mbo*II restriction site and converts Trp627 to Cys (W627C). Digestion of genomic DNA PCR fragments with *Mbo*II indicated that the patient and the father were heterozygous for this missense mutation in exon 12. Failure to find cDNAs from patient RNA which did not contain this mutation suggested that the maternal mutation leads to greatly reduced synthesis or reduced stability of mRNA from the mutant allele. In order to identify the maternal mutation, it was necessary to analyze genomic sequences. This approach was complicated by the finding of multiple unprocessed pseudogenes and/or closely related genes. Using PCR with a panel of human/rodent hybrid cell lines, we found that these pseudogenes were present over chromosomes 5–7, 20, and 22 and the Y chromosome. Conditions were defined which allowed us to amplify and characterize genomic sequences for the true β-glucuronidase gene despite this background of related sequences. The patient proved to be heterozygous for a second mutation, in which a C→T transition introduces a termination codon (R356STOP) in exon 7. The mother was also heterozygous for this mutation. Expression of a cDNA containing the maternal mutation produced no enzyme activity, as expected. Expression of the paternal mutation in COS-7 cells produced a surprisingly high (65% of control) level of activity. However, activity was 13% of control in transiently transfected murine MPS VII cells. The level of activity of this mutant allele appears to correlate with the level of overexpression, suggesting that high concentrations of mutant monomers can drive the folding and tetramerization of mutant enzyme to produce an active and stable enzyme.

Introduction

Human β-glucuronidase (E.C.3.2.1.31) is a lysosomal enzyme composed of four identical subunits that is involved in the stepwise degradation of glucuronic acid–containing glycosaminoglycans (Brot et al. 1978). Deficiency of this enzyme results in the accumulation, in lysosomes, of partially degraded glycosaminoglycans containing glucuronic acid residues at their nonreducing termini. In humans, this enzyme deficiency produces the clinical genetic disorder mucopolysaccharidosis type VII (MPS VII, or Sly syndrome) (Hall et al. 1973; Sly et al. 1973).

In the approximately 25 cases of MPS VII reported, patients exhibited a wide range of phenotypic heterogeneity (Lee et al. 1985). To date, only two mutations have been identified in studies of three Japanese patients (Tomatsu et al. 1990, 1991). Two patients, a 6-year-old female and a 24-year-old male, were homozygous for the missense mutation Ala619→Val and

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demonstrated a phenotype consisting of hepatomegaly, umbilical herniation, and slight bone deformities. The 24-year-old male also demonstrated unusual facies, short stature, striking coarse metachromatic granules, and mental retardation. The third patient was homozygous for the mutation Arg382→Cys and demonstrated umbilical herniation, severe bone deformities, and short stature. This patient had normal intelligence and facies and exhibited no hepatomegaly or abnormal granules. These two reports mentioned that complex Southern digest patterns suggested the presence of β-glucuronidase pseudogenes in the genome.

The subject of this study was the first individual diagnosed with this disorder. He had an intermediate phenotype and was reported to have less than 2% residual levels of β-glucuronidase activity in fibroblasts (Hall et al. 1973; Sly et al. 1973; Bachinsky 1991). He presented in infancy with hernias and hepatosplenomegaly, showed mild mental retardation (IQ = 60) and moderately severe and progressive skeletal deformities involving the thorax, spine, hip, and knee joints, and cardiac valvular abnormalities, and died suddenly at age 19 years, possibly from a cardiac arrhythmia. Southern blot patterns were complex and provided no evidence for gross deletions within the β-glucuronidase locus. Northern analysis revealed that mRNA was normal in size but somewhat reduced in quantity (Bachinsky 1991). These data suggested the presence of one or more mutations affecting the specific activity of the enzyme and possibly the level of mRNA. We report here the paternal mutation, which was identified from PCR analysis of cDNA from the patient. Absence of a recognizable maternal allele in cDNA clones from the patient made it necessary to analyze the genomic DNA for the maternal mutation, which was complicated by the presence of multiple pseudogenes.

An important goal of this study was to define conditions for analysis of β-glucuronidase mutations that could only be demonstrated by PCR of genomic sequences because one or both alleles do not produce mRNA. This goal required us first to identify and characterize the pseudogenes. Several unprocessed pseudogenes were identified by PCR and assigned to specific human chromosomes by PCR analysis of DNA from a panel of human/rodent hybrid cell lines. Then, conditions were established which circumvent the problems raised by the multiple pseudogenes and which permit genomic analysis of patient DNA. Use of these conditions allowed us to identify the maternal mutation in the genomic DNA, and this procedure should be generally applicable for the mutational analysis of genomic sequences of the human β-glucuronidase gene.

Material and Methods

Material

Restriction enzymes were purchased from Promega and New England Biolabs. *Taq* polymerase and buffer were purchased from Promega for amplification of genomic DNA, and an RNA PCR kit was purchased from Cetus for cDNA synthesis and amplification. M13mp18, M13mp19, DEAE dextran, and deoxynucleotides for PCR and sequencing were from Pharmacia LKB. Sequenase was from United States Biochemicals. The vector PCR1000 was from Invitrogen. A kit for site-directed mutagenesis was purchased from Amersham. RNAzol was from Cinna Biotecx. A kit for extraction of genomic DNA was purchased from Applied Biosystems. Low-melting-temperature agarose was from FMC. Oligo-(dT) cellulose and chloroquine were from Sigma.

Cell Lines

Fibroblasts for patient C.R. (GM0121), his mother (GM2074), and his father (GM1850), as well as genomic DNA from a panel of human/rodent somatic cell hybrid lines, were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). Fibroblasts were grown in Dulbecco's minimal essential medium (DMEM) containing 15% FBS. COS-7 cells were grown in DMEM containing 5% FBS. Line 3521 cells (fibroblasts from the abdominal wall of the MPS VII mouse) were established in this laboratory by J.H.G.

RNA PCR

RNA was isolated from patient fibroblasts with RNAzol according to a procedure described by Chomczynski and Sacchi (1987), poly(A)+ RNA was selected over oligo-(dT) cellulose (Jacobson 1987), and 0.1 μg of poly(A)+ RNA was used as a template for reverse transcriptase. First-strand cDNA synthesis for the 337f/1007r and 965f/1978r reactions was primed with oligo-(dT), while that of the −20f/422r reaction was primed with random hexamers by using the Cetus RNA PCR kit. PCR was for 45 cycles using 20 pmol of each primer. Cycle conditions were denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 2 min, with a 7-min denaturation at 93°C before the first cycle and a 7-min extension at 72°C after the final cycle. The −20f/422r reaction

contained a 3:1 molar ratio of c7dGTP:dGTP to eliminate secondary structure (McConlogue et al. 1988).

Amplification of Genomic DNA

Genomic DNA was extracted from fibroblasts by using a kit from Applied Biosystems. One microgram of genomic DNA was amplified with 20 pmol of each primer by using *Taq* polymerase and buffer from Promega. Denaturation was for 1 min at 96°C (for exons 1 and 2) or 94°C (for all other reactions). Annealing conditions were as follows: 2 min at 61°C for exon 1; 2 min at 60°C for exons 2, 5, 6–7, and 11 (primers 11f and 11r); 2 min at 68°C for exon 3; 1 min at 58°C for exons 8, 9, and 10; 2 min at 58°C for exon 4; 2 min at 65°C for exon 11 (primers 11fa and 11r) and exons 2–4 (primers 40f and 41r); and 1 min at 55°C for exon 12. All extensions were at 72°C for 1 min (exon 9), 2 min (exons 1, 2, 3, 4, 8, 10, 11, and 12), or 3 min (exons 2–4, 5, and 6–7). Reactions were for 30 cycles (exons 2–4, 5, 6–7, 8, 10, 11, and 12), 35 cycles (exons 1, 3, and 4), 40 cycles (exon 9), or 45 cycles (exon 2). A 7-min extension at 72°C followed the final cycle of all reactions.

Subcloning and Sequencing

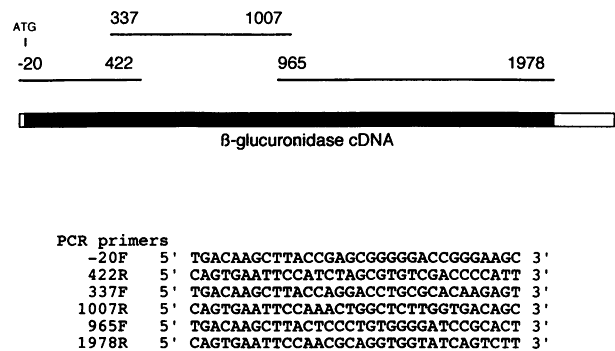
PCR fragments were isolated using low-melting-temperature agarose. The exon 6–7 PCR fragment was, on occasion, treated with *Sty*I prior to gel isolation to restrict pseudogene clones, and clones representing the expressing alleles were thereby enriched by isolation of the uncut species. The 337f/1007r and 965f/1978r PCR products were cut with *Eco*RI and *Hin*dIII and subcloned into M13 (Messing 1983) for sequencing. Other fragments were either subcloned directly into PCR1000 or were made blunt-ended with T4 DNA polymerase (Sambrook et al. 1989) and were subcloned into the *Sma*I site of M13. Sequencing was done using the dideoxy chain-termination method (Sanger et al. 1977). In some cases, dGTP was replaced with dITP to sequence through compressions.

PCR of Human/Rodent Somatic Cell Hybrids

One-half microgram of genomic DNA from each hybrid line was amplified using 20 pmol each of primer pairs 40f/41r (exons 2–4) and 6f/7r. One-quarter microgram of DNA was used with the 11f/11r primer pair. PCR conditions were the same as those described above for genomic DNA.

Expression

cDNAs containing the W627C and R356STOP mutations were constructed by site-directed mutagenesis

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PCR primers
  -20F   5' TGACAAGCTTACCGAGCGGGGGACCGGGAAGC 3'
  422R   5' CAGTGAATTCCATCTAGCGTGTCGACCCCATT 3'
  337F   5' TGACAAGCTTACCAGGACCTGCGCACAAGAGT 3'
 1007R   5' CAGTGAATTCCAAACTGGCTCTTGGTGACAGC 3'
  965F   5' TGACAAGCTTACTCCCTGTGGGGATCCGCACT 3'
 1978R   5' CAGTGAATTCCAACGCAGGTGGTATCAGTCTT 3'
```

Figure 1 Strategy for RNA PCR of human β-glucuronidase. *Top,* First-strand cDNA amplified in three segments encompassing the coding region. Untranslated areas are shown unblackened. *Bottom,* Sequences of the PCR primers. *Hind*III and *Eco*RI sites were constructed into forward and reverse PCR primers, respectively, to facilitate subcloning.

(Nakamaye and Eckstein 1986) using a kit supplied by Amersham. Normal and mutant cDNAs were subcloned into the expression vector pJC119 (Sprague et al. 1983) for expression in COS-7 cells (Gluzman 1981). COS cells were transfected by using a DEAE dextran procedure (Lopata et al. 1984) followed by a 3-h treatment with 100 μM chloroquine (Luthman and Magnusson 1983). Cells were harvested 72 h after transfection in 0.5% sodium deoxycholate (DOC), and extracts and media were assayed fluorometrically with 4-methylumbelliferyl-β-glucuronide (Glaser and Sly 1973). Normal and mutant cDNAs were also subcloned into the mammalian expression vector pMPSVEH (Artelt et al. 1988) and were used to transfect murine MPS VII cells (line 3521 from the abdominal wall). The same transfection procedure was used, except that treatment with DEAE dextran was for 1.5 h.

Results

Identification of the Paternal Mutation

RNA from patient fibroblasts was amplified in three segments, as shown in figure 1. At least 10 clones of each fragment were sequenced, and these clones were representative of at least two PCR reactions, so that allelic mutations could be distinguished from *Taq* polymerase misincorporations. A single mutation was found in exon 12 of the cDNA, which changed Trp627 to a Cys residue (W627C). This G→T transversion destroys an *Mbo*II restriction site. This mutation was found in 13 of 13 PCR clones from this region, suggest-

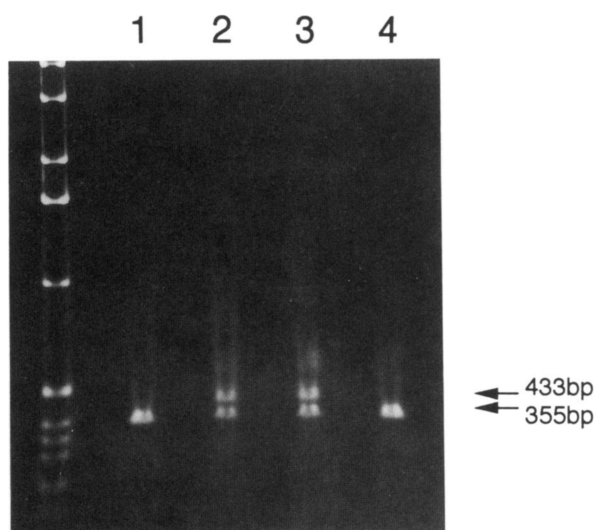


Figure 2 PCR amplification showing paternal inheritance of the W627C mutation. Genomic DNA from a normal control (lane 1), the patient (lane 2), the patient's father (lane 3), and the patient's mother (lane 4) was amplified by PCR with primers 71f and 72r, and the PCR products were digested with *Mbo*II. Normal PCR fragments contain an internal *Mbo*II site which gives rise to a 355-bp digestion product. This site is destroyed by the W627C mutation, resulting in a 413-bp fragment.

ing that the patient might be homozygous for this allele. However, differences in β-glucuronidase–specific activity and RNA levels in fibroblasts from the parents of the patient had suggested that the patient might be a compound heterozygote (Bachinsky 1991). To investigate the inheritance of this allele, we amplified exon 12 of the genomic DNA from the patient and both parents and digested the PCR products with *Mbo*II (fig. 2). Normally, the 433-bp fragment is converted to a 355-bp fragment on digestion with *Mbo*II; and the mutation prevents this conversion. Both the patient and his father are heterozygous for this mutation, having one allele which is sensitive to *Mbo*II and one which is not. The PCR fragment of the mother is completely digested with *Mbo*II, indicating that she does not carry this mutation. This result was confirmed by sequencing several clones of each PCR fragment. Roughly half of the clones from the father contained the mutation, while no clones from the mother had the mutation. These results clearly showed that the patient is not homozygous for the W627C mutation. We concluded that amplification of genomic DNA from the patient would be necessary to identify the maternal mutation, since this allele is apparently not present in the patient's mRNA.

An incidental finding was that 1 of 13 RNA PCR clones from the 965f/1978r fragment appeared to represent the product of a previously unidentified alternative splicing event for β-glucuronidase. Exons 9–11 are missing, and exon 8 has been precisely spliced to exon 12. The coisolation of this clone with the 1-kb full-length PCR fragment was surprising, since it is 397 bp shorter than the full-length fragment. No band of this size is detectable by ethidium bromide staining following PCR of this region. This event, which presumably occurs at a low frequency, destroys the reading frame and would likely produce an inactive protein. The skipping of exon 6, which produces an in-frame deletion of 51 amino acids in human β-glucuronidase, occurs at a much higher frequency (Oshima et al. 1987).

Unprocessed Pseudogenes

PCR amplification of genomic DNA revealed a number of apparent unprocessed pseudogenes for human β-glucuronidase, which made mutational analysis of genomic sequences appear quite formidable. For example, when exon 11 was amplified initially using intronic primers, 19 of 20 PCR products cloned and sequenced were of pseudogene origin. These findings made it necessary to characterize the magnitude of the problem for each exon and then to design ways to circumvent it.

Pseudogenes of several different types were identified during amplification of exons 2–4, 3, 6–7, and 11. One type of pseudogene was identified by using exonic primers that amplify exons 2–4, another type by using intronic primers surrounding exon 3 only, one type by using intronic primers for exons 6–7, and three types by using intronic primers for exon 11. Sequence comparisons with the normal coding sequence for each of these exons are shown in figure 3. The pseudogenes share a high (70%–95%) degree of identity within the coding region of the β-glucuronidase gene. The sequences identified by amplification of exon 3, exons 2–4, and exons 6–7 are clearly from pseudogenes, as they contain frameshifts and stop codons which would prevent the production of an active enzyme. However, the sequences identified by amplification of exon 11 do not contain frameshifts or stop codons. It is therefore possible that these sequences could represent copies of closely related functional genes.

The chromosomal localization of the pseudogenes was investigated using DNAs from a panel of human/rodent somatic cell hybrid lines. Except for two cases, each line contains a single human chromosome on a

```
exon 2 wild type   CAGTTCCCTCCAGCTTCAATGACATCAGCCAGGACTGGCGTCTGCGGCATTTTGTCGGCTGGGTGTGGTACGAA
exon 2-4 pseudo    .G.................C......G......G......G...............A.....T.......T...

CGGGAGGTGATCCTGCCGGAGCGATGGACCCAGGACCTGCGCACAAGAGTGGTGCTGAGGATTGGCAGTGCCCATTCCTATGCCATCGTG
..........C.....T...........T..........T..............................-...C.T.............


exon 3 wild type   TGGGTGAATGGGGTCGACACGCTAGAGCATGAGGGGGGCTACCTCCCCTTCGAGGCCGACATCAGCAACCTGGT
exon 2-4 pseudo    .....A...............G..........A.................T................G....T.
exon 3 pseudogene  ...........T...A................----.----------------..T..........G.T....

CCAGGTGGGGCCCCTGCCCTCCCGGCTCCGAATCACT-ATCGCCATCAACAACACACTCACCCCCACCA-------CCCTGCCACCAGGGACC
........A.........T....AC..T.C.....-..................G.........--.--------....-.-........
............T..-.....T.C...T.CG.....A...AA.....GT..T.TG....A.....AG.GGGGGT................
...TGC...A

ATCCAATACCTGACTGACACCTCCAA
...TGTC.A-....C.....-..-.-
...TGC...A....CA..........


exon 4 wild type GTATCCCAAGGGTTACTTTGTCCAGAACACATATTTTGACTTTTTCAACTACGCTGGACTGCAGCGGTCTGTACTT
exon 2-4 pseudo  ...............................G.C........C....G......A.......T.T.....C.G...

CTGTACACGACACCCACCACCTACATCGATGACATC
..A...........C.................G..


exon 6 wild type   GTGCAGCTGACTGCACAGACGTCACTGGGGCCTGTGTCTGACTTCTACACACTCCCTGTGGGGATCCGCACTGTG
exon 6 pseudogene ...TG..............A.............-.---.........................C...........

GCTGTCACCAAGAGCCAGTTCCTCATCAATGGGAAACCTTTCTATTTCCACGGTGTCAACAAGCATGAGGATGCGGAC
..C......G...................G.........................C........................


exon 7 wild type   ATCCGAGGGAAGGGCTTCGACTG-GCCGCTGCTGGTGAAGGACTTCAACCTGCTTCGCTGGCTTGGTGCCAACGC
exon 7 pseudogene ....A.-...........A....T...............................T...-.......-......A.

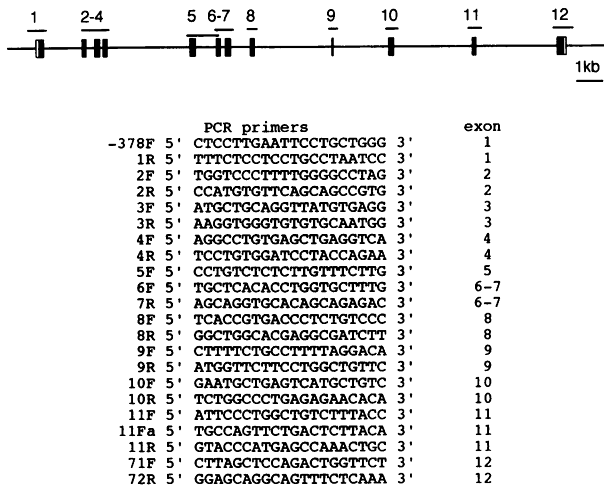
TTTCCGTACCAGCCACTACCCCTATGCAGAGGAAGTGATGCAGATGTGTGACCGCTATGGGATTGTGGTCATCGATGAGTGTCCCGGCGTGGG
C...T.C...........T.....CA.G.....GA..C.......A...T....G.............................T.CT.....

CCTGGCGCTGCC
....AT......


exon 11 wild type         GATCCACCTCTGATGTTCACTGAAGAGTACCAGAAAAGTCTGCTAGAGCAGTACCATCTGGGTCTGGAT
exon 11 pseudo (type 1) ...................G.......G.....G................................CA..
exon 11 pseudo (type 2) ...................G......C..........................................
exon 11 pseudo (type 3) .G...........................G.......C.........................

CAAAAACGCAGAAAATATGTGGTTGGAGAGCTCATTTGGAATTTTGCCGATTTCATGACTGAACAGT
.................C..................C.........................A.C....
.......T.........C..................C.........................A.C....
.................C...............T..CG......C...........C....ATG....
```

Figure 3 Sequence comparisons of the β-glucuronidase pseudogenes with the wild-type gene. Comparisons are shown for exons 2, 3, 4, 6, 7, and 11. Residues of identity are indicated by dots, while gaps are shown by dashes.



PCR primers				exon
-378F	5'	CTCCTTGAATTCCTGCTGGG	3'	1
1R	5'	TTTCTCCTCCTGCCTAATCC	3'	1
2F	5'	TGGTCCCTTTTGGGGCCTAG	3'	2
2R	5'	CCATGTGTTCAGCAGCCGTG	3'	2
3F	5'	ATGCTGCAGGTTATGTGAGG	3'	3
3R	5'	AAGGTGGGTGTGTGCAATGG	3'	3
4F	5'	AGGCCTGTGAGCTGAGGTCA	3'	4
4R	5'	TCCTGTGGATCCTACCAGAA	3'	4
5F	5'	CCTGTCTCTCTTGTTTCTTG	3'	5
6F	5'	TGCTCACACCTGGTGCTTTG	3'	6–7
7R	5'	AGCAGGTGCACAGCAGAGAC	3'	6–7
8F	5'	TCACCGTGACCCTCTGTCCC	3'	8
8R	5'	GGCTGGCACGAGGCGATCTT	3'	8
9F	5'	CTTTTCTGCCTTTTAGGACA	3'	9
9R	5'	ATGGTTCTTCCTGGCTGTTC	3'	9
10F	5'	GAATGCTGAGTCATGCTGTC	3'	10
10R	5'	TCTGGCCCTGAGAGAACACA	3'	10
11F	5'	ATTCCCTGGCTGTCTTTACC	3'	11
11Fa	5'	TGCCAGTTCTGACTCTTACA	3'	11
11R	5'	GTACCCATGAGCCAAACTGC	3'	11
71F	5'	CTTAGCTCCAGACTGGTTCT	3'	12
72R	5'	GGAGCAGGCAGTTTCTCAAA	3'	12

Figure 4 Strategy and intronic primer sequences for PCR of human β-glucuronidase from genomic DNA. *Top,* Exons amplified individually by using intronic primers, except for exons 6–7, which were amplified together, and for exon 5, which was amplified by using an exonic reverse primer in exon 6 (1007r; fig. 1). Exons 2, 3, and 4 were amplified individually or together. *Bottom,* Sequences of the intronic PCR primers.

mouse or hamster background. PCR amplification of genomic DNA from hybrid lines and sequencing of PCR fragments revealed that the exon 2–4 pseudogene was located on chromosome 22 and that the exon 6–7 pseudogene was located on chromosome 5. To our surprise, each of the three pseudogenes for exon 11 was found to be located on two different chromosomes. The first pseudogene (called "type 1") was found on chromosomes 5 and 6, the second (type 2) on chromosomes 20 and 22, and the third (type 3) on chromosome 7 and the Y chromosome. The exon 3 pseudogene appears to be located on the Y chromosome.

Having identified the pseudogenes and having defined the magnitude of the problem they posed for mutational analysis, we adopted the following strategy for genomic analysis of patient DNA. When intronic primers were used to amplify exons 2–4 individually, only 10%–20% of the PCR clones were of pseudogene origin, and these exons could be analyzed without difficulty. With exons 6–7 and 11, 55% and 95% of the clones were of pseudogene origin, respectively. The exon 6–7 pseudogene contained a *Sty*I site not found in the expressing alleles. To minimize the cloning of pseudogene products, the PCR fragments from exons 6–7 were digested with *Sty*I prior to gel isolation. The exon 11 pseudogene problem was eliminated by designing a new PCR primer (primer 11fa) such that all of the pseudogenes contained mismatches with the primer at or near its 3′ end. Preferential amplification of the β-glucuronidase alleles by PCR using this primer was achieved by raising the annealing temperature by 5°C. Having established these conditions, we pursued the mutational analysis of the patient's genomic DNA.

Identification of the Maternal Mutation

β-Glucuronidase sequences from genomic DNA were amplified using the intronic primers shown in figure 4. In addition, the 1007r primer located in exon 6 was used in conjunction with oligo 5f to amplify exon 5. No mutations were found in the 200-bp region immediately 5′ to the coding region which has previously been shown to contain the promoter elements essential for expression (Shipley et al. 1991). This indicated that the maternal mutation which presumably affects RNA stability was located elsewhere. The only mutation found was a C→T transition in exon 7, which creates a

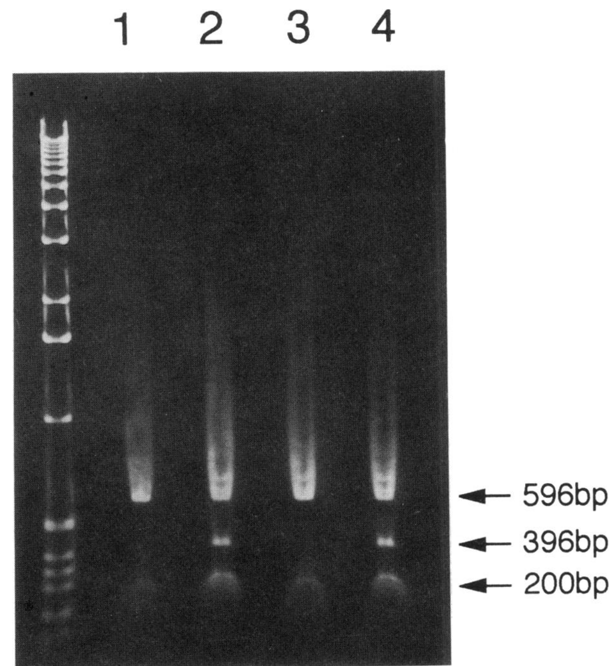


Figure 5 PCR amplification showing maternal inheritance of the R356STOP mutation. Genomic DNA from a normal control (lane 1), the patient (lane 2), the patient's father (lane 3), and the patient's mother (lane 4) was amplified by PCR with primers 6f and 7r, and the PCR products were digested with *Bgl*II. Normal PCR products of 556 bp contain no *Bgl*II site. The R356STOP mutation creates a *Bgl*II site which gives rise to fragments of 396 bp and 200 bp.

Table 1

Expression of Normal and Mutant Enzymes in Mammalian Cells

	β-Glucuronidase Activity	
Construct	Units/mg Cell Protein Minus Endogenous	% of Wild Type
COS cells:		
Wild type	831	100
W627C	536	65
R356STOP	0	0
3521 cells:		
Wild type	158	100
W627C	21	13

NOTE.—COS cells and 3521 murine MPS VII cells were transfected with the indicated constructs, as described in Material and Methods. The cells were harvested 72 h posttransfection in 1 ml of 0.5% DOC. Samples were assayed for β-glucuronidase activity by using 4-methylumbelliferyl-β-D-glucuronide. One unit is the amount of activity that releases 1 nmol of 4-methylumbelliferone/h. Values reflect the average of two plates.

new *Bgl*II site and changes Arg356 to a stop codon. Exons 6–7 were amplified from genomic DNA from both parents of the patient to identify the origin of this allele. When this mutation is present, digestion of the 596-bp PCR product with *Bgl*II produces fragments of 396 bp and 200 bp (fig. 5). Fragments indicating the presence of this mutation are seen in PCR products from the patient and his mother, but not in PCR products from the father or a normal control. The restriction digest results were confirmed by sequencing. Sequencing of several exon 6–7 PCR clones from the mother revealed that half of the clones contain the mutation. None of the exon 6–7 PCR clones from the father have the mutation. No other mutations in the coding region were identified in genomic sequences from the patient, nor were any potential splice junction mutations found. These data indicate that the patient is a compound heterozygote, having inherited a missense mutation from his father and a nonsense mutation from his mother. The maternal mutation presumably reduces the stability of the β-glucuronidase mRNA and accounts for the underrepresentation of this allele in the cDNA from patient fibroblasts (Atweh et al. 1988; Baserga and Benz 1988; Daar and Maquat 1988; Lim et al. 1989; Urlaub et al. 1989; Cheng et al. 1990; Kadowaki et al. 1990; Barker and Beemon 1991; Genovese et al. 1991; Mashima et al. 1992).

Expression of the Mutant Alleles in Mammalian Cells

Mutant cDNAs were constructed using site-directed mutagenesis and were subcloned into the expression vector pJC119 for expression in COS-7 cells. Enzyme activities are shown in table 1. As expected, the cDNA containing the maternal mutation which creates a stop codon in the middle of the coding sequence (R356STOP) produces no enzyme activity above background. In contrast, the paternal W627C mutant produces 65% of wild-type levels in transfected COS cells. One possible explanation for this unexpectedly high level of activity on transient expression is that the activity we observe is due to newly synthesized enzyme which has not yet reached lysosomes and because the mutant enzyme may become inactive only after delivery to lysosomes. To investigate this possibility, we studied the fate of normal and mutant enzymes endocytosed by cultured MPS VII fibroblasts. For these studies, normal and mutant W627C enzyme secreted from transfected COS cells was used for uptake. Patient fibroblasts were allowed to take up enzyme for a period of 24 h, at which time the medium was replaced with DMEM, and the stability of the enzyme was followed over a period of 3 d by measurement of the intracellular enzyme activity. The endocytosed W627C enzyme activity was no less stable than the endocytosed wild-type enzyme over this period (i.e., there was no loss in activity), indicating that the secreted mutant enzyme was stable in fibroblast lysosomes (data not shown).

Another potential explanation for the higher-than-expected activity of the mutant enzyme is that the mutation is one which disrupts folding of the enzyme, and this defect can be overcome by the enormous enzyme concentration related to its gross overproduction in COS cells. Less than 10% of the cells are transfected, and cells which are transfected each contain up to 200,000 copies of plasmid (Mellon et al. 1981). The local overproduction of enzyme monomers may lead, by mass action, to folding and tetramerization of mutant enzyme which would not fold properly and would not tetramerize when expressed at physiological levels. Supporting this hypothesis was the observation that less activity was seen compared with that of the normal enzyme when both were expressed at much lower levels in murine MPS VII cells. The vector used for this experiment was pMPSVEH, which still expresses, at a reasonably high level, from the myeloproliferative sarcoma virus promoter. Unlike SV40 origin-containing vectors which are used for their ability to replicate in

COS cells, pMPSVEH does not replicate in mammalian cells. For this reason, transfected cells contain a far lower plasmid copy number. In this system, expression of the mutant enzyme gives rise to 13% of the activity produced by the wild-type enzyme (table 1). Since only a small percentage of the cells are transfected, the transfected cells are still likely producing relatively high local levels of enzyme. We suspect that expression of mutant enzyme in stably selected clonal lines at single-copy level may result in still lower levels of residual activity, levels approaching those seen in patient fibroblasts (i.e., below 2% of normal).

Discussion

The American patient, in whom β-glucuronidase deficiency was first shown to be the basis for his mucopolysaccharidosis storage disease, proved to be a compound heterozygote, having inherited a missense mutation from his father and a nonsense mutation from his mother. Both mutations were quite interesting, but for different reasons.

The paternal mutation, which was seen in every RNA PCR clone, is a missense mutation, in exon 12, which changes Trp627 to Cys and destroys an *Mbo*II restriction site. *Mbo*II digestion of genomic PCR fragments from patient and parental DNAs demonstrated the paternal inheritance of this allele, which was confirmed by sequencing. Overexpression of enzyme containing the W627C mutation produced a very interesting result —namely, an unexpectedly high level of intracellular and secreted enzyme activity (65% of normal control). This activity was as stable as the wild-type enzyme, after endocytosis by fibroblasts. Transient lower-level expression of the mutant enzyme in murine MPS VII cells resulted in considerably less activity relative to the level expressed following transfection of the normal cDNA (13% of normal control). This phenomenon of higher-than-expected activity of a mutant enzyme has also been observed with mutations in another lysosomal enzyme, hexosaminidase A, in which deleterious mutations producing negligible activity in fibroblasts gave higher levels of enzyme activity when overexpressed in COS cells (Mahuran and Brown 1991). The model proposed to explain this result was that residual activities from mutations which adversely affect the folding constant, K_{μ}, are enhanced in overexpression systems because of the high concentration of newly synthesized unfolded protein in the endoplasmic reticulum (Mahuran and Brown 1991). Since β-glucuronidase exists as a tetramer, overexpression may influence the kinetics of formation of active tetramers. The locally high concentration of monomers may drive the tetramerization of a mutant enzyme which otherwise might not tetramerize at physiological levels of expression. A third possibility is that the cysteine introduced in the W627C mutant may participate in aberrant disulfide bond formation to a greater extent when it is expressed at low levels in patient fibroblasts, possibly because disulfide isomerase is present in excess under these conditions.

The maternal mutation is a nonsense mutation in exon 7 which creates a *Bgl*II site in the genomic DNA. It is of interest because it appears to affect RNA stability, since it was not identified in any RNA PCR clones. There is ample precedence for nonsense mutations which affect RNA stability. Nonsense mutations in β-globin (Atweh et al. 1988; Baserga and Benz 1988; Lim et al. 1989), triose phosphate isomerase (Daar and Maquat 1988; Cheng et al. 1990), dihydrofolate reductase (Urlaub et al. 1989), the insulin receptor (Kadowaki et al. 1990), the immunoglobulin heavy chain (Genovese et al. 1991), the Rous sarcoma virus gag gene (Barker and Beemon 1991), and the ornithine aminotransferase gene (Mashima et al. 1992) have also been shown to affect RNA stability. This effect has also been seen with frameshift mutations which create nonsense codons (Baumann et al. 1985; Fojo et al. 1988; Lim et al. 1989). One proposed explanation for this effect that nonsense mutations have on mRNA levels is that translation may be coupled to RNA processing and/or to nuclear export of the RNA (Urlaub et al. 1989; Cheng et al. 1990). Another possible explanation is that premature translational termination results in detachment of the mRNA from the ribosome, which exposes the RNA to endogenous nucleases and thus affects half-life and turnover of the RNA.

Several unprocessed pseudogenes for β-glucuronidase were identified by using PCR primers which amplify exons 2–4, exon 3 only, exons 6–7, and exon 11. One type of pseudogene was found for exons 2–4, one for exon 3, and one for exons 6–7. Three types of pseudogenes were found for exon 11. The exon 2–4 pseudogene and the exon 6–7 pseudogene were found to be located on chromosome 22 and chromosome 5, respectively, by PCR amplification of genomic DNA from a panel of human/rodent somatic cell hybrid lines. Each of the three exon 11 pseudogenes was found to be located on two chromosomes. Type 1 was found on chromosomes 5 and 6, type 2 on chromosomes 20 and 22, and type 3 on chromosome 7 and the Y chromo-

some. Therefore, at least six unprocessed pseudogenes for β-glucuronidase are scattered throughout the genome.

Sequences sharing homology to other exons may also exist, but none was identified with our intronic PCR primers. For example, a clone isolated from a cDNA library shares a very high degree of homology with exons 5, 9, 10, and 11 of β-glucuronidase (Sukegawa et al. 1987). Although all of the β-glucuronidase pseudogenes contain several base changes which would result in amino acid substitutions, only the exon 3, exons 2–4, and exons 6–7 pseudogenes contain termination codons which would necessarily preclude the production of an active enzyme. The exon 11 pseudogenes contain uninterrupted open reading frames and may actually represent closely related functional genes. They could code for other glucuronidases, such as the glucuronidase which acts only on nonsulfated glycosaminoglycans (Nakamura et al. 1990) or the endoglucuronidase which degrades chondroitin sulfate (Takagaki et al. 1988). They may also code for another glycosylhydrolase, as suggested by the fact that human β-glucuronidase shares 32% identity with *Escherichia coli* β-galactosidase over a stretch of 155 amino acids (Oshima et al. 1987).

The pseudogenes initially posed a formidable problem for mutational analysis of exonic and surrounding sequence from genomic DNA. In fact, 95% of clones originally identified for exon 11 were of pseudogene origin. This problem was circumvented by designing a PCR primer which maximized mismatches with the pseudogenes at or near the 3′ end and by defining conditions under which use of this primer amplified the expressing β-glucuronidase alleles but not the pseudogenes. The exon 6–7 pseudogene problem was alleviated by digestion of the PCR product with *Sty*I prior to gel isolation. This enzyme digests the pseudogene but not the normal gene. These modifications should have general application in identification of mutations from genomic DNA in MPS VII patients when one or both alleles are not represented in the mRNA.

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