Mucopolysaccharidosis Type VII: Characterization of Mutations and Molecular Heterogeneity

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Summary

We identified two different exonic point mutations causing β-glucuronidase (βGl) deficiency in two Japanese patients with mucopolysaccharidosis type VII (MPSVII). Enzyme assay of lysates of the lymphocytes and cultured fibroblasts showed little residual activity. The βGl-specific mRNA levels were normal, as determined by northern blot analysis. Mutated cDNA clones, including the entire coding sequence, were isolated using the polymerase chain reaction (PCR) products derived from βGl-deficient fibroblasts. Sequence analysis of the full-length mutated cDNAs showed C→T transitions, which resulted in a single Ala^{619}→Val change (case 1, a 24-year-old male) and a Arg^{382}→Cys change (case 2, a 7-year-old female). The former change was revealed by a loss of the cleavage site for the Fnu4HI in the mutated cDNA. On the basis of the loss of Fnu4HI restriction site, the patient (case 1) was a homozygote with this mutation. The mutational change in patient 2 was confirmed by direct sequencing and by demonstrating heterozygosity for the mutation in her parents. The Ala^{619}→Val and Arg^{382}→Cys mutations each disrupt a different domain which is highly conserved among human, rat, and *Escherichia coli* βGls. Each of these two amino acid changes reduced the βGl activity of the corresponding mutant βGl expressed following transfection of COS cells with expression vectors harboring the mutated cDNAs.

Introduction

Mucopolysaccharidosis type VII (MPSVII) is a genetic autosomal recessive trait characterized by accumulation of undegraded glycosaminoglycans due to a markedly decreased activity of β-glucuronidase (β-Gl; E.C.3.2.1.31). The clinical manifestations are unusual facies, mental retardation, short stature, hepatomegaly, bony deformities, glycosaminoglycan excretion, and striking coarse metachromatic granules. The phenotype is various and heterogeneous. The heterogeneity in phenotypes and the clinical variations relate to several different mutations, each of which may have a various

effect on the activity of the enzyme. MPSVII has not been definitely classified. The nucleotide sequences of a cDNA encoding human βGl have been determined (Oshima et al. 1987). We found that the βGl gene of an 8-year-old Japanese female with MPSVII showed the single Ala^{619}→Val change to be the cause of this enzyme defect (Tomatsu et al. 1990).

The development of the polymerase chain reaction (PCR) (Saiki et al. 1985) has facilitated a replication of cDNA, and DNA sequencing and library constructions and screening are obviated. We now report the isolation and characterization of two mutated BGl cDNA, determined using the PCR. The molecular heterogeneity of MPSVII and the expression of the mutated cDNAs in transfected COS-7 cells are described. Direct sequencing procedures were used for confirmation of mutations, and a family study was done for one patient.

Material and Methods

Material

Restriction endonucleases were purchased from Takara Shuzo (Kyoto) and Nippon Gene (Toyama, Japan). T4DNA ligase, polIK, T4DNA polymerase, reverse transcriptase, and *Thermus aquaticus* (*Taq*) polymerase were obtained from Takara Shuzo and BRL (Gaithersburg, MD). All sequencing primers for pUC plasmids and specific oligoprimers were synthesized using an automated DNA synthesizer (model 380A; Applied Biosystems).

Cell Lines and Culture

The first patient (case 1) was a 24-year-old Japanese male with clinical features of unusual facies, hepatomegaly, umbilical herniation, slight bone deformity, mental retardation, short stature, and striking coarse metachromatic granules. There is consanguinity in his family history, but the family refused to provide tissue/blood samples for analysis. The second patient (case 2) was a 7-year-old Japanese female with clinical features of umbilical herniation, severe bone deformity, short stature, normal intelligence, no hepatomegaly, normal facies and no abnormal granules. There is no record of consanguinity in the history of this patient. In both these patients with MPSVII, levels of βGl activity in lymphocytes and cultured fibroblasts ($\sim$2% of normal values) were undetectable. The βGl-deficient and control fibroblasts were maintained in 5% CO_2 in MEM (minimum essential medium) supplemented with 10% FCS.

Labeling of DNA

Labeling was performed with a Multiprimer kit and [α-^{32}P] dCTP (3,000 Ci/mmol) from Amersham (Buckinghamshire). Oligodeoxynucleotide primers were labeled at the 5' terminus with [γ-^{32}P] ($\sim$5,000 Ci/mmol; Amersham).

RNA and cDNA Preparation

A total of approximately 2×10^7 cells were washed twice with PBS and extracted as total RNA by guanidinium thiocyanate (Chromczynski and Sacchi 1987). Five micrograms of total RNA was mixed with 5.0 μg of random hexamer primers (pd [N$_e$]; Takara) in a total volume of 15.5 μl, including 20 units of RNase inhibitor (RNasin; Pharmacia) and 4 μl of reverse transcriptase buffer (250 mM Tris-HCl pH 8.3(37°C), 50 mM MgCl$_2$, 250 mM KCl, 15 mM DTT). The mixture was kept at 65°C for 15 min, then centrifuged.

An additional 20 units of RNasin, 2.0 μl of a 25-mM mix of all four dNTPs, and 2.0 μl of Moloney murine leukemia virus reverse transcriptase (200 units/μl; BRL) were added to bring the total volume to 20 μl. Reaction mixtures were incubated at 37°C for 1 h. About 50% of the products of one reverse transcriptase reaction were annealed to 50 pmol each of sense primers and antisense primers. After heating of the reaction mixture, amplification was carried out for 40 cycles as follows: 7 min at 93°C to inactivate the proteases, 1 min denaturation at 94°C, 2 min annealing at 54°C, and 3 min extension at 72°C.

Northern Blotting

Total RNA transfer onto nylon membrane filters (Amersham) and hybridization were performed using a glyoxal method and a 1.2% agarose.

### Subcloning and Sequencing of PCR-amplified Products

PCR-amplified products were blunted by T$_4$ polymerase with dNTPs and subcloned into an *Sma*I site of pUC vector. Otherwise, they were digested at restriction-enzyme sites that had been incorporated into the inside of cDNA fragments or the priming oligonucleotides (fig. 1) and ligated into pUC vectors. This DNA was then used to transform the JM101 or JM109 strain of *Escherichia coli*. Plasmid DNA was extracted from transformants, and double-stranded pUC plasmids containing the inserts were sequenced, in both strands, by using the dideoxynucleotide chain termination method of Hattori and Sakaki (1986).

Direct Sequencing

PCR products were purified and extracted in 20 μl of 1 mM EDTA, 10 mM Tris-HCl, pH8.0, by using a Gene Clean kit. The extracted fragments were dried and dissolved in a final volume of 6 μl of H_2O. Direct sequencing reactions were carried out by mixing 6.0 μl of DNA template and 2.0 μl of 5 $\times$ sequenase buffer and by heating the mixture to 95°C for 2 min and spinning it for purposes of condensation. After annealing of the template mixture to 2.0 μl of 5'-labeled primer, the sequencing reactions were carried out using the termination mixtures of a Sequenase kit (USB; Cleveland) and were analyzed using a 6% polyacrylamide gel.

Transfection

Transfection of COS-7 cells was carried out according to a method described elsewhere (Oshima et al. 1987). In brief, 8 μg of DNA/ml of MEM containing 200 μg/ml of DEAE-dextran (mol wt >1 $\times$ 10^6; Phar-
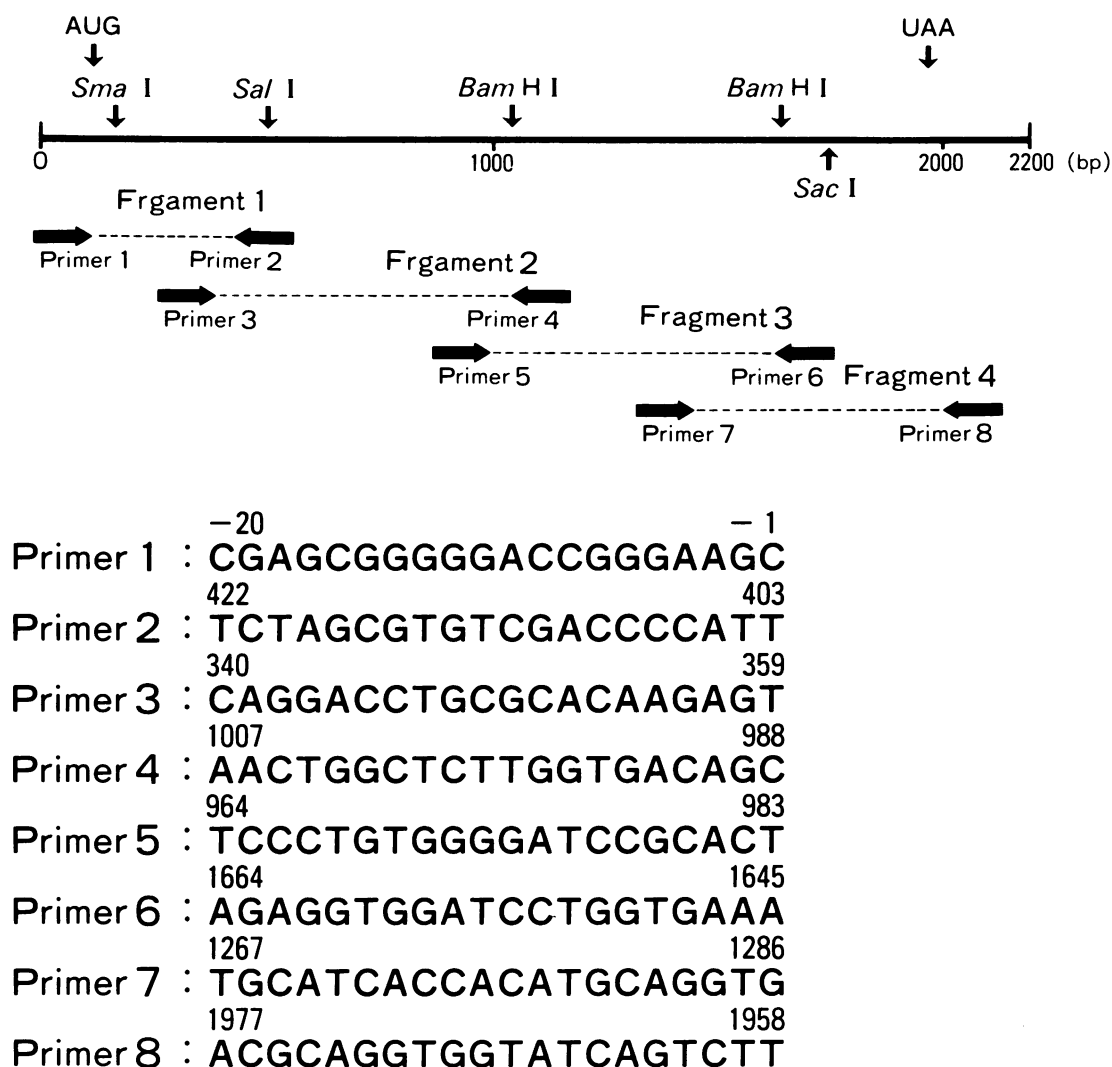
Figure I   Strategy for PCR amplification of human βGl cDNA. The complementary positions of each primer to the βGl cDNA are shown by arrows beneath the solid line; and primers were used for PCR and DNA sequencing. The first and last bases of the primers (5' to 3') that are complementary to either the antisense (primers 1, 3, 5, and 7) or sense (primers 2, 4, 6, and 8) sequence of the βGl cDNA are numbered according to the cDNA sequence, where the A of the first in-frame ATG is designated base number 1.

macia) and 50 mM Tris-HCl pH7.4 was added to 5 × 10⁵ cells/dish for 8 h at 37°C. The medium was aspirated, and, after several washes with MEM, 2 ml of fresh medium containing 10% FCS and 100 μM chloroquine were added, and the preparation then was maintained at 37°C for 5 h. The cells were then washed with MEM and incubated in 5 ml of MEM containing fresh 10% FCS.

Enzymatic Activity

Seventy-two hours after transfection the cells were washed twice with PBS and were collected with a rubber policeman in 1 ml of PBS. The suspension was cen-

trifuged for 2 min at 2,000 g in an International centrifuge, and the cells were precipitated and frozen immediately at −70°C. Samples containing a known amount of protein were tested for enzymatic activity with 4-methylumbelliferyl-β-glucuronide as a substrate, according to a method described elsewhere (Sukegawa and Orii 1985).

Results

Northern Blotting

Total RNA from the patients was electrophoresed, transferred onto nylon membrane filters, and hybrid-
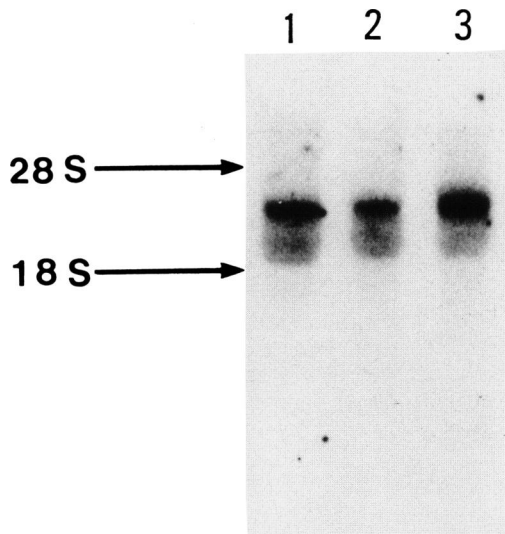
Figure 2   Northern blot of total RNA. The northern blot contains 160 μg of total RNA from each of a normal subject (lane 1), case 1 (lane 2), and case 2 (lane 3).

ized with labeled 2.2-kb cDNA. There were broadened bands of about 2.3 kb in total RNAs from both the patients and normal subjects, and there was no difference, in either quality or quantity, among the samples (fig. 2). Subsequent rehybridization with a labeled β-actin probe (Takara Shuzo) showed that there was no major RNA degradation in the samples and that β-actin mRNA was present at equivalent levels in normal and MPSVII cell strains. The 2.3-kb band seems to represent the full length of mRNA for βGl. The normal transcripts in these patients rule out a promoter mutation as cause of the enzyme defect.

βGl cDNA Amplification

The amplification of four fragments (fragments 1–4) containing the entire peptide coding region of human βGl cDNA (1,956 nucleotides) was achieved by using as starting material the equivalent of 5 μg of total cellular RNA. Each cDNA amplification fragment was subcloned into pUC vectors. Sequencing of the cloned cDNAs was performed entirely in both directions (sense and antisense strands) by using universal primers and βGl-specific oligo primers. In case 1, a single point mutation was revealed at amino acid 619, but in case 2 double point mutations were evident at amino acids 382 and 649. All point mutations are from a C→T transition. A C→T transition in case 1 substitutes Val for Ala^{619} (GC̲G→GT̲G) and deletes an Fnu4HI site (GCAGC̲). The C→T transitions in case 2 cause

Arg^{382}→Cys and Pro^{649}→Leu. To rule out an erroneous replication by the enzyme Taq polymerase, more than five independent clones were sequenced and a new set of experiments was carried out when these nucleotide changes were detected. The experiment was repeated at least twice, starting from cDNA synthesis of the total RNA from the patients. The results clearly showed that cDNAs contained a single Ala^{619}→Val change for case 1 and both Arg^{382}→Cys and Pro^{649}→Leu changes for case 2. The amino acid sequence of the entire βGl-mutated cDNAs was normal, except for these changes, and no other nucleotide changes were found on sequencing the entire cDNA from either patient. The Ala^{619}→Val change was detected by a loss, in the mutated cDNA, of the cleavage site for the enzyme Fnu4HI. On the basis of this observation, the patient (case 1) was shown to be a homozygote with this mutation (fig. 3, top). Moreover, direct sequencing around the mutation also showed that this patient was homozygous for this mutation (fig. 3, bottom). To prove that both Arg^{382}→Cys and Pro^{649}→Leu changes are indeed present in the cDNA from case 2, direct sequencing was performed, including a family study. The results revealed that the patient (case 2) was homozygous for these substitutions, a finding consistent with Mendelian segregation (figs. 4). When the same set of experiments was done to amplify and sequence the cDNAs from 20 Japanese unrelated normal controls, the former change was never evident.

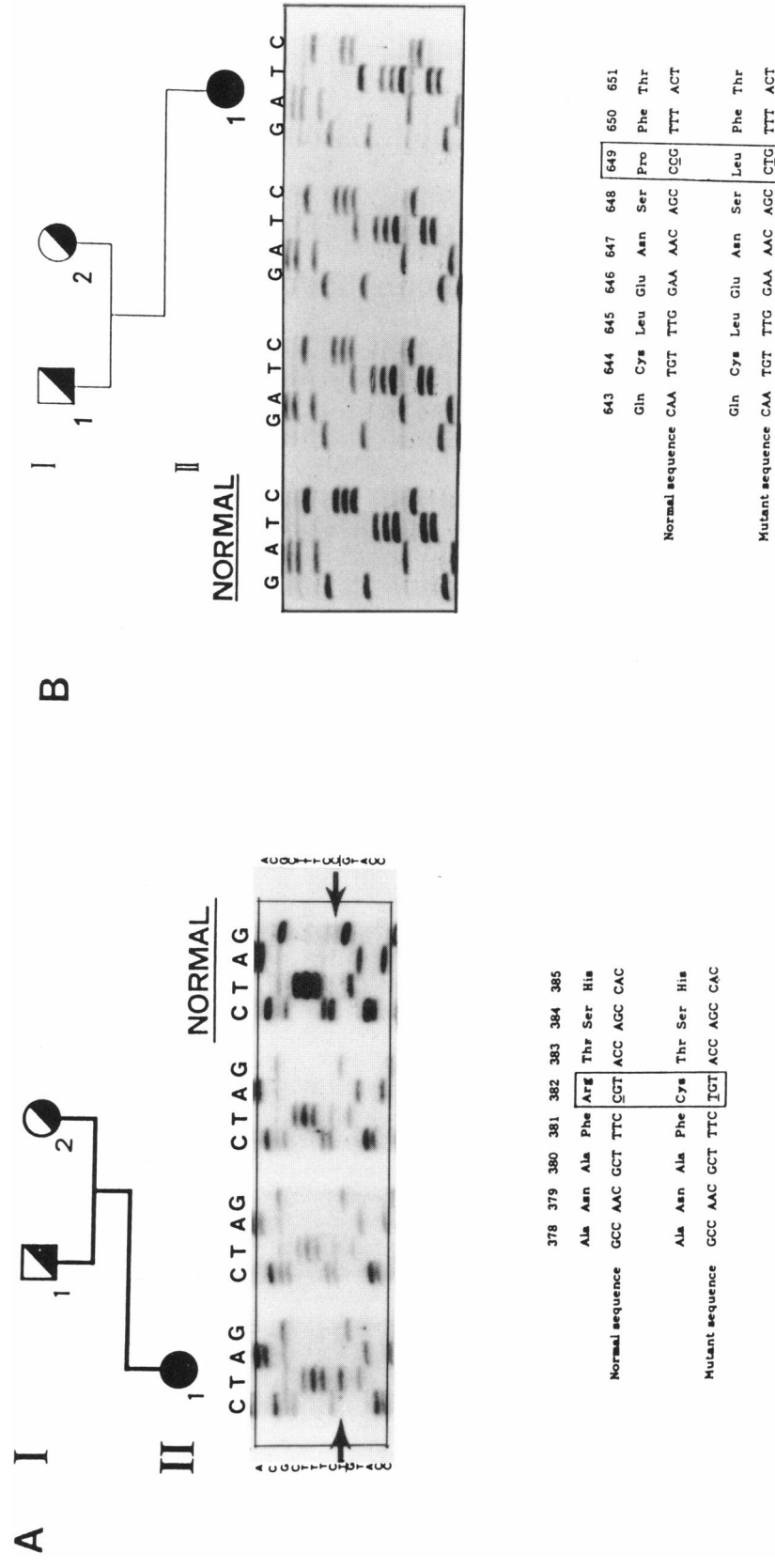## Transient Expression of the Mutated cDNAs in COS-7 Cells

To determine whether the amino acid substitutions described above would alter the enzymatic activity of the βGl protein, the mutated cDNAs were coupled to the SV40 late promoter in an SV shuttle vector (pSVL; Pharmacia). The normal βGl cDNA was subcloned into the SmaI site of pSVL, designated pSVL(βGN). The corresponding fragment from the mutated cDNAs and these expression vectors were designated pSVL(Ala^{619}→Val), pSVL(Arg^{382}→Cys), and pSVL (Pro^{649}→Leu) according to the protocol described above. The pSVL(Ala^{619}→Val) was constructed as described elsewhere (Tomatsu et al. 1990), and pSVL(Arg^{382}→Cys) was constructed by replacement of the BamHI fragment from pSVL(βGN) by the BamHI-digested fragment 3 (fig. 1) including the Arg^{382}→Cys mutation. After the fragment 4 including the Pro^{649}→Leu mutation was filled in by PolIK, it was ligated into the SmaI site of pUC13, and the SacI frag-
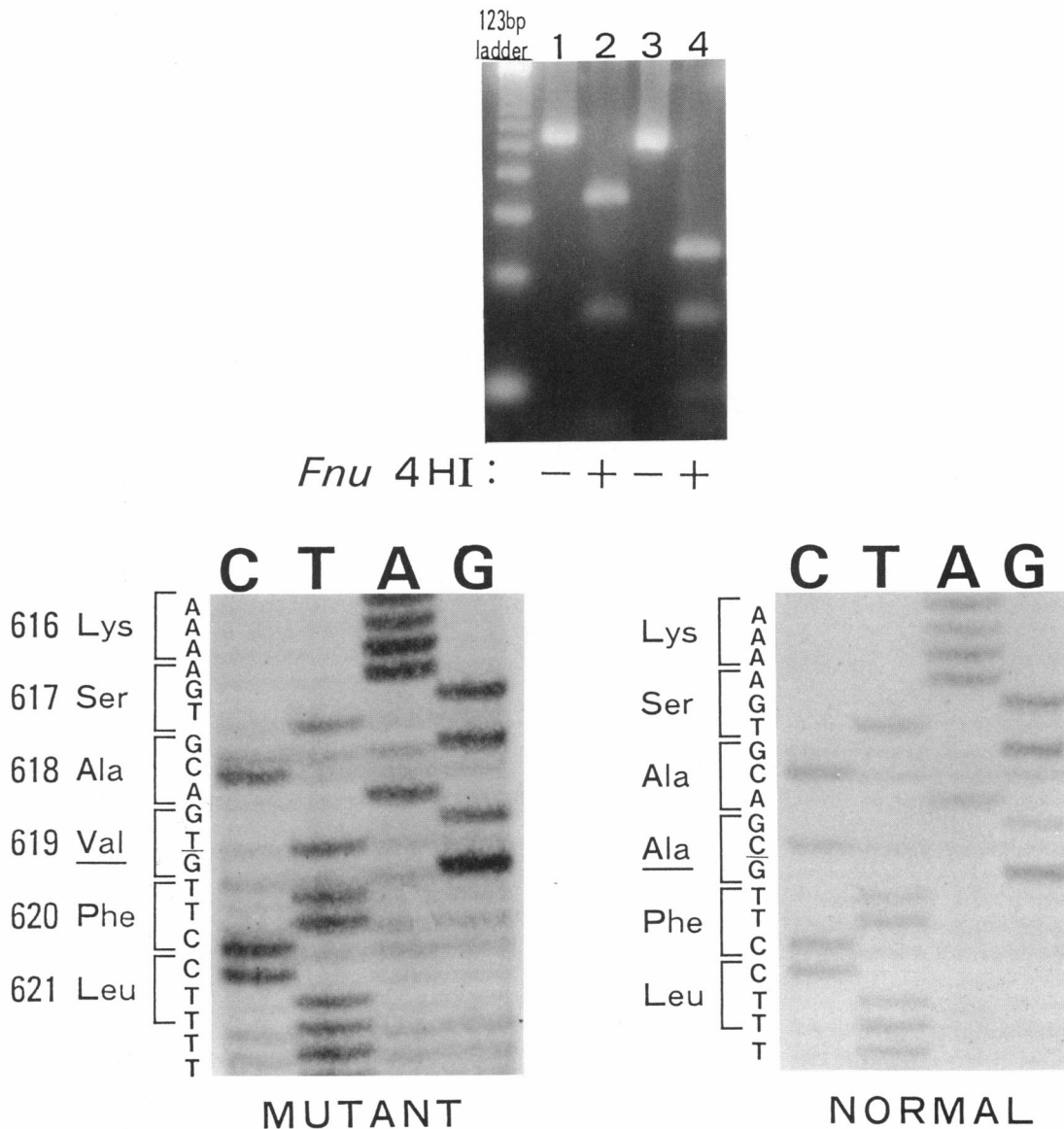
Figure 3   *Fnu*4HI restriction assay and direct sequencing for Ala[619]→Val mutation. *Top,* Ethidium bromide–stained 2% agarose gels, showing electrophoretic separation of fragments obtained after digestion of PCR-amplified cDNA segment with (+) or without (−) *Fnu*4HI. The cDNA samples, 711-bp fragments, were produced using primers 7 and 8. The 711-bp fragment is divided into 295-, 196-, 125-, 92-, and 3-bp fragments after digestion in normal subjects. On the other hand, the 295- and 125-bp fragments are replaced with the 420-bp fragment because of loss of the *Fnu*4HI restriction site in an affected patient. The cDNA samples were derived from a patient (lanes 1 and 2) and from a normal control (lanes 3 and 4). The first lane is a 123-bp ladder (Bethesda Research Laboratories). *Bottom,* Nucleotide and corresponding amino acid alteration, shown by direct sequencing of an MPSVII patient. The nucleotide and amino acid of the mutant site are underlined.

ment from pSVL(βGN) was replaced by the mutated *Sac*I fragment derived from this plasmid. As shown in table 1, in COS-7 cells transfected with the normal cDNA, βGl activity increased about 20 times, compared with the intracellular βGl activity. In cells transfected

with the pSVL(Ala[619]→Val) and pSVL(Arg[382]→Cys) there was no normal increase in βGl activity, while in cells transfected with the pSVL(Pro[649]→Leu) there was the same increase as seen in the normal cDNA. The efficiency of transfection was confirmed by extraction

A

NORMAL

	378	379	380	381	382	383	384	385
	Ala	Asn	Ala	Phe	Arg	Thr	Ser	His
Normal sequence	GCC	AAC	GCT	TTC	CGT	ACC	AGC	CAC
	Ala	Asn	Ala	Phe	Cys	Thr	Ser	His
Mutant sequence	GCC	AAC	GCT	TTC	TGT	ACC	AGC	CAC

B

NORMAL

	643	644	645	646	647	648	649	650	651
	Gln	Cys	Leu	Glu	Asn	Ser	Pro	Phe	Thr
Normal sequence	CAA	TGT	TTG	GAA	AAC	AGC	CCG	TTT	ACT
	Gln	Cys	Leu	Glu	Asn	Ser	Leu	Phe	Thr
Mutant sequence	CAA	TGT	TTG	GAA	AAC	AGC	CTG	TTT	ACT

Figure 4 Direct sequencing analysis of $Arg^{382} \rightarrow Cys$ (A) and $Pro^{649} \rightarrow Leu$ (B) changes in the family of case 2. The pedigree chart of the family is included above both the sequencing results (■ and ◐ = carriers; ● = homozygote). The amplified cDNA samples were derived from total RNA of normal and family-member mutant fibroblasts by using primers 5 and 6 for the $Arg^{382} \rightarrow Cys$ change and primers 7 and 8 for the $Pro^{649} \rightarrow Leu$ change. Direct sequencing was performed with the labeled primers 5 and 8, respectively. The arrows show the mutation sites, and the sequences have a T (mutant) substituted for a C (normal). The nucleotide alteration and the corresponding amino acid alteration are indicated beneath the respective sequencing gels. The nucleotide and amino acid substitutions are boxed.

Table I

Expression of Human β-GI in Transfected COS-7 Cells

	CELL EXTRACT	
Plasmid	Total No. of Units	Heat Resistance (nmol/mg protein/h)
pSVL	34	15
pSVL(βGN)	283	263
pSVL (case 1: Ala^{619}→Val)	45	26
pSVL (case 2: Arg^{382}→Cys)	35	12
pSVL (case 2: Pro^{649}→Leu)	250	235

Note.—COS cells were transfected with pSVL(βGN) or with pSVL(Ala^{619}→Val), pSVL(Arg^{382}→Cys), or pSVL(Pro^{649}→Leu), as described in the text. βGl activity was assayed 72 h after transfection. The enzyme activity was defined as the activity releasing 1 nmol 4-methylumbelliferone/h.

of each expression vector DNA from the transfected COS cells and by subsequent Southern blot analysis (data not shown).

Discussion

A molecular analysis of MPSVII was done by cDNA cloning using PCR. We identified two different point mutations causing βGl deficiency. Each of these mutations had a dramatic effect on the activity of the enzyme, determined on the basis of transfection into COS cells by mutated cDNAs. Northern blotting analysis of these two MPSVII patients showed βGl mRNA of an apparently normal size and quality, thereby suggesting an exonic point mutation rather than a promoter mutation. Southern blot analysis of the total genomic DNA after 12 kinds of restriction-enzyme digestions indicated that several pseudogenes were present in the haploid genome (data not shown). Thus, it is most difficult to identify a mutation by genomic DNA analysis until the complete gene structure has been elucidated.

One nucleotide change noted in a 24-year-old affected male was C→T transition causing a substitution of Val for Ala^{619} (G$\underline{C}$G→G$\underline{T}$G; fig. 3) and a loss of an Fnu4HI restriction site. The two changes in the 7-year-old girl are also C→T transitions, causing substitutions of Cys for Arg^{382} ($\underline{C}$GT→$\underline{T}$GT; fig. 4A) and of Leu for Pro^{649} (C$\underline{C}$G→C$\underline{T}$G; fig. 4B), but there were no changes in restriction sites. The family study revealed both parents to be heterozygotes for an allele containing both of these changes. Of the identified Ala^{619}→Val, Arg^{382}→Cys, and Pro^{649}→Leu changes, it was confirmed that only the former two changes were linked to the inactivation of enzyme activity, because in COS cells transfected with these mutated cDNAs in a pSVL

expression vector, there was no normal increase. It is interesting that, following transfection with the cDNA encoding the enzyme with the Ala^{619}→Val change, there was a slight increase in the activity (about twice that of the endogeneous activity). At 96 and 120 h post-transfection, the same increase in activity was detected. No increase was detected with pSVL(Arg^{382}→Cys) at any time (unpublished data). Thus these two mutations have different effects on the enzyme. Moreover, the Pro^{649}→Leu change caused no reduction in the enzyme activity. Both Arg^{382}→Cys and Pro^{649}→Leu changes were inherited from the parents, a finding consistent with a Mendelian segregation. It is possible to say that in this patient the Pro^{649}→Leu change and the Arg^{382}→Cys change are on the same allele. The above three changes were C→T transitions within CpG doublets. In the past few years the role of CpG dinucleotides has been shown to be an important cause of the development of spontaneous point mutations because of subsequent deamination of methylcytosine to thymine, leading to a C→T transition (Barker et al. 1984). The amino acid changes, each of which altered a CpG doublet, and the type of all the nucleotide transitions observed are consistent with an accidental deamination event on a coding 5-methylcytosine.

Computerized molecular modeling of the normal and mutant enzyme proteins around Ala^{619}→Val mutation showed that this change is unlikely to cause significant alterations in the secondary structure and electric charge (data not shown). Ala^{619} is in a center of a hydrophobic region relatively near the putative glycosylation site, and the change does not change the charge. On the other hand, the Arg^{382}→Cys substitution possesses a more negative charge, and the change in charge may explain why this mutation produces no increase of the

Table 2

Amino Acid Sequences Near the Mutation Site of Various βGl's

	AMINO ACID[a]								
β-GLUCURONIDASE	378	379	380	381	382	383	384	385	REFERENCES
HUMAN βGl (normal)	Ala	Asn	Ala	Phe	Arg	Thr	Ser	His	Oshima et al. 1987
βGl (Arg[382]→Cys)	Ala	Asn	Ala	Phe	<u>Cys</u>	Thr	Ser	His	Present study
Rat βGl	Ala	Asn	Ser	Phe	Arg	Thr	Ser	His	Nishimura et al. 1986
Murine βGl	Ala	Asn	Ser	Phe	Arg	Thr	Ser	His	Gallagher et al. 1988
Escherichia coli βGl	Ala	Asn	Ser	Tyr	Arg	Thr	Ser	His	Jefferson et al. 1986

[a] Residue numbers are based on the amino acid sequence of human βGl (Oshima et al. 1987).

enzyme activity (data not shown). The state of the sulfhydryl group of Cys that substituted for Arg[382] requires more attention by using mutant protein analysis such as SDS-PAGE, because it is possible that Cys is involved in the formation of an inter- or intramolecular disulfide bond between or within abnormal molecules.

It is notable that both Arg[382] and Ala[619] are invariant in the βGl of all the species tested to date—and, moreover, that, as represented in table 2, Arg[382] is present within a comparatively conserved region of the molecule, as is Ala[619] (Tomatsu et al. 1990). Although the results presented here identify Arg[382] and Ala[619] as important in human βGl, it was not determined whether these sites are directly involved in specific binding or catalytic interactions or are critical only for maintaining both the structure of the binding site and protein stability. Site-directed mutagenesis may clarify related mechanisms involved in the relationship of structural to functional change. The finding that these mutations in MPS VII patients are located at different domains of the enzyme can explain the phenotypic heterogeneity found in this disease. Moreover, it will serve as a useful tool for diagnosis, family study, and accurate genotyping of MPS VII patients.

Acknowledgments

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