Am. J. Hum. Genet. 53:881–888, 1993

Mutations in Acid β-Galactosidase Cause GM_1-Gangliosidosis in American Patients

R.-M. Boustany,* W.-H. Qian,† and K. Suzuki‡

*Division of Pediatric Neurology and †Department of Pediatrics, Duke University, Durham; and ‡Brain and Development Research Center, University of North Carolina, Chapel Hill

Summary

We describe four new mutations in the β-galactosidase gene. These are the first mutations causing infantile and juvenile GM_1-gangliosidosis to be described in American patients. Cell lines from two patients with juvenile and from six patients with infantile GM_1-gangliosidosis were analyzed. Northern blot analysis showed the acid β-galactosidase message to be of normal size and quantity in two juvenile and four infantile cases and of normal size but reduced quantity in two infantile cases. The mutations are distinct from the Japanese mutations. All are point mutations leading to amino acid substitutions: $Lys^{577} \rightarrow Arg$, $Arg^{590} \rightarrow His$, and $Glu^{632} \rightarrow Gly$. The fourth mutation, $Arg^{208} \rightarrow Cys$, accounts for 10 of 16 possible alleles. Two infantile cases from Puerto Rico of Spanish ancestry are homozygous for this mutation, suggesting that this allele may have come to South America and North America via Puerto Rico. That these mutations cause clinical disease was confirmed by marked reduction in catalytic activity of the mutant proteins in the Cos-1 cell expression system.

Introduction

Genetic deficiency of lysosomal β-galactosidase in humans causes both GM_1-gangliosidosis, a progressive neurological disorder, and Morquio disease type B (Morquio B), the latter manifesting as generalized skeletal dysplasia with connective tissue involvement (O'Brien 1989; Wray and Boustany 1989). GM_1-gangliosidosis has three clinical phenotypes: (1) an infantile variant: onset is at birth to age 2 mo, with hypotonia, coarse features, organomegaly, and cherry red spots and with death by 1 to 2 years of age; (2) a juvenile variant: onset is between 1 and 2 years of age, with a lag in motor and cognitive development and lack of dysmorphic features (survival can be until the teen years); and (3) an adult variant: onset is late, with a progressive extrapyramidal disorder due to local deposition of GM_1-gangliosidosis in the caudate nucleus. Oshima (1988) and Morreau et al. (1989) published the normal cDNA sequence for acid β-galactosidase, allowing the mutational analysis of the β-galactosidase gene in patients with GM_1-gangliosidosis and Morquio B (Nishimoto et al. 1991; Oshima et al. 1991). We have examined the acid β-galactosidase gene in eight North American and South American patients and have identified four new mutations.

Received January 27, 1993; final revision received May 26, 1993.

Address for correspondence and reprints: Rose-Mary Boustany, Division of Pediatric Neurology, Duke University Medical Center, Box 3533, Durham, NC 27710.

0002-9297/93/5304-0012$02.00

Patients, Material, and Methods

Patients

Eight patients from North American and South American families were included in the study. Three of the families were of Hispanic descent. The clinical findings are summarized in table 1.

Commercial Materials

Bethesda Research Laboratories (Gaithersburg, MD), Boehringer-Mannheim (Indianapolis), New England Biolabs (Beverly, MA), Pharmacia LKB Biotechnology (Piscataway, NJ), Promega (Madison, WI), Fisher-Scientific (Fair Lawn, NJ), Sigma Chemical (St. Louis), and United States Biochemical (Cleveland) were the main sources for standard enzymes, reagents, and other supplies. Radioisotopes were purchased from DuPont New England Nuclear (Boston).

Table 1

Phenotype/Genotype Summary of North American and South American Mutations

Case	Type (sex)	Origin	Age at Onset	At Diagnosis: Age	CRS[a]	Large Liver	Bony Changes	HC[b,c]	mRNA (size, amount)	Alleles
1	Juvenile (F)	English-German	12 mo	2½ years	–	–	–	↓[d]	+, +	R590H/R208C (U/S)
2	Infantile (F)	English	4 mo	20 mo	+	–	–	N	+, +	K577R/R208C (W/S)
3	Infantile (M)	Eastern European-English	?	2½ years	–	–	+	N	+, +	R208C/? (S/?)
4	Infantile (F)	Puerto Rican	10 mo	2 years	–	–	+	N	+, ↓[d]	R208C/R208C (S/S)
5	Infantile (F)	Mexican	6 mo	11 mo	+	–	+	↓[d]	+, +	K577R/R208C (W/S)
6	Juvenile (F)	English-Scottish	4½ years	8 years	–	–	–	N	+, +	E632G/R208C (Z/S)
7	Infantile (M)	Puerto Rican	6 mo	1 year	?	+	+	N	+, ↓[d]	R208C/R208C (S/S)
8	Infantile (F)	English	5 mo	13 mo	–	–	–	↓[d]	+, +	R208C/? (S/?)

[a] Cherry red spot.
[b] Head circumference.
[c] N = normal.
[d] Diminished.

Cell Culture

Skin fibroblasts were grown in Dulbecco's modified Eagle minimum essential medium supplemented with 10% FCS at 37°C in 5% CO_2 atmosphere. Normal control cell lines and disease control cell lines were selected from the stock in our laboratory (disease controls included infantile GM_2-gangliosidosis and late-infantile and juvenile neuronal ceroid lipofuscinosis).

RNA Preparation and Northern Analysis

Total RNA was isolated from 20 100-mm Petri dishes by the single-step method using phenol saturated with water, sodium acetate, and guanidium isothiocyanate. Thirty micrograms of total RNA were electrophoresed in a 1% agarose, 0.6 M formaldehyde gel and were transferred to a nylon membrane (Biotrans; ICN Biomedicals, Costa Mesa, CA). The full-length normal human β-galactosidase cDNA probe and the 1.1-kb β-actin probe were labeled by nick-translation with [α-^{32}P]dCTP. Hybridization and subsequent washings were done according to instructions supplied by the manufacturer. The β-galactosidase cDNA probe was stripped from the filter by boiling for 5 min in 100 mM Tris-HCl pH 7.5, 1 mM EDTA, 1% SDS. The same filter was reprobed by using the labeled β-actin cDNA.

Reverse Transcription and PCR Amplification

Five micrograms of total RNA were reverse transcribed by using 0.5 μg oligo dT/μg RNA and 45 units of AMV reverse transcriptase. The resultant first-strand cDNA was amplified in two segments, divided at the internal *Eco*RI site with two pairs of primers. The 5′ and 3′ segments were 800 bp and 1,500 bp, respectively. Thirty cycles of amplification were carried out in 100 μl of the reaction mixture with 2.5 units of *Taq* polymerase (Perkin Elmer Cetus, Norwalk, CT). Denaturing temperature was 94°C for 1 min, annealing temperature was 60°C for 2 min, and extension temperature was 72°C for 3 min.

Subcloning and Sequence Analysis

The amplified segments were digested, separated by gel electrophoresis, and directly extracted from the gel bands, using phenol and phenol-chloroform prior to subcloning into pGEM-3zf(−) vector (Promega, Madison, WI). At least four clones per fragment per patient were analyzed. Double-stranded sequencing was

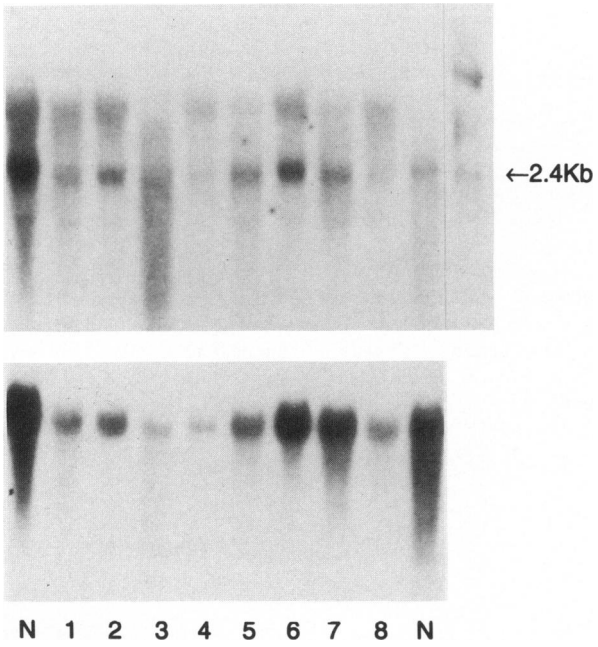

Figure 1 Northern blot analysis. Thirty micrograms of total RNA sample isolated from fibroblasts were electrophoresed on a denaturing formaldehyde agarose gel. *Top,* Probe was the full-length human β-galactosidase cDNA. *Bottom,* Blot was then stripped and reprobed with the β-actin cDNA to allow for comparative quantitation. The numbers below the diagram indicate case numbers as in table 1. Lanes N, Normal controls. The arrow indicates the position of the normal β-galactosidase mRNA at 2.4 kb. The message was of normal size in all cases but was diminished in quantity in two infantile patients (cases 4 and 7). All other patients showed mRNA of normal size and quantity.

carried out with the Sequenase version 2.0 kit (United States Biochemicals, Cleveland), ^{35}S-labeled ATP (DuPont New England Nuclear, Boston), and SP6, T-7, and M-13 universal primers.

Genotype Determination with Allele-specific Oligonucleotides

Genomic DNA was isolated from four 100-mm Petri dishes according to standard methods. Approximately 500 ng of genomic DNA were used as template for PCR amplification of target exons, with appropriate primers. Thirty cycles of amplification were carried out, with a denaturing temperature of 94°C for 1 min, an annealing temperature of 50°C for 2 min, and an extension temperature of 72°C for 3 min. Extension during the last cycle was carried out for 10 min. The amplified segments were purified by phenol-chloroform extraction and were precipitated in ethanol. Fifteen nanograms of the PCR products were dot blotted onto Gene Screen Plus membrane, using a BRL Hybri-Dot manifold (Bethesda Research Labs, Gaithersburg, MD). The membrane was baked for 2 h at 80°C and hybridized at 45°C for 2 h in 6 × SSC, 5 × Denhardt's solution, 10 mM sodium phosphate, 0.5% SDS, and 100 μg heat-denatured herring sperm DNA/ml. Hybridization was at 45°C overnight in the same solution with 2×10^6 cpm appropriate oligonucleotide probe labeled at the 5′ terminus with [γ-^{32}P]ATP and T_4 polynucleotide kinase. The membrane was washed at room temperature in 2 × SSC for 10 min twice, then at 5°C lower than the melting temperature for the oligonucleotide in 2 × SSC, 0.1% SDS for 15 min, and was finally autoradiographed after exposure at −70°C.

Direct Sequencing of Amplified Target Exons from Genomic DNA

Amplified exons were gel purified, and 0.1 pmol of purified PCR product was denatured by heating 2 min at 95°C, then snap cooled in a dry ice/ethanol bath for 1 min and annealed to 0.5 pmol of primer for 30 min. Double-stranded sequencing was carried out as previously described, with Sequenase and ^{35}S-labeled ATP.

Expression of Catalytic Activity in the Cos-1 Cell System

Transformed African green monkey kidney cells (Cos-1) were purchased from the American Type Culture Collection (Rockville, MD). cDNAs, each bearing only one of the four identified mutations, were constructed from the normal cDNA and appropriate fragments of the mutant cDNA in the pGEM-3zf(−) vector. The cDNA inserts containing the entire coding region (normal β-galactosidase or one of four mutant β-galactosidase cDNAs) were excised from the plasmid by *Xba*I/*Sac*I double digestion and subcloned into the pSVL eukaryotic expression vector (Pharmacia LKB Biotechnology, Piscataway, NJ). Transfection of the Cos-1 cells was achieved by utilizing liposomes (Lipofectin Reagent, BRL). The period of transfection was 5 h with 0.5 μg of plasmid DNA and 50 μl of the Lipofectin Reagent/35-mm dish. At 72 h after transfection the cells were harvested for enzyme assay.

Enzyme Assay

Acid β-galactosidase activity was determined from homogenates of cultured fibroblasts or transfected Cos-1 cells by using 4-methylumbelliferyl β-D-galactoside (Koch-Light Labs, Colnbrook Bucks, U.K.) (Suzuki 1987). Protein determination was by the microassay

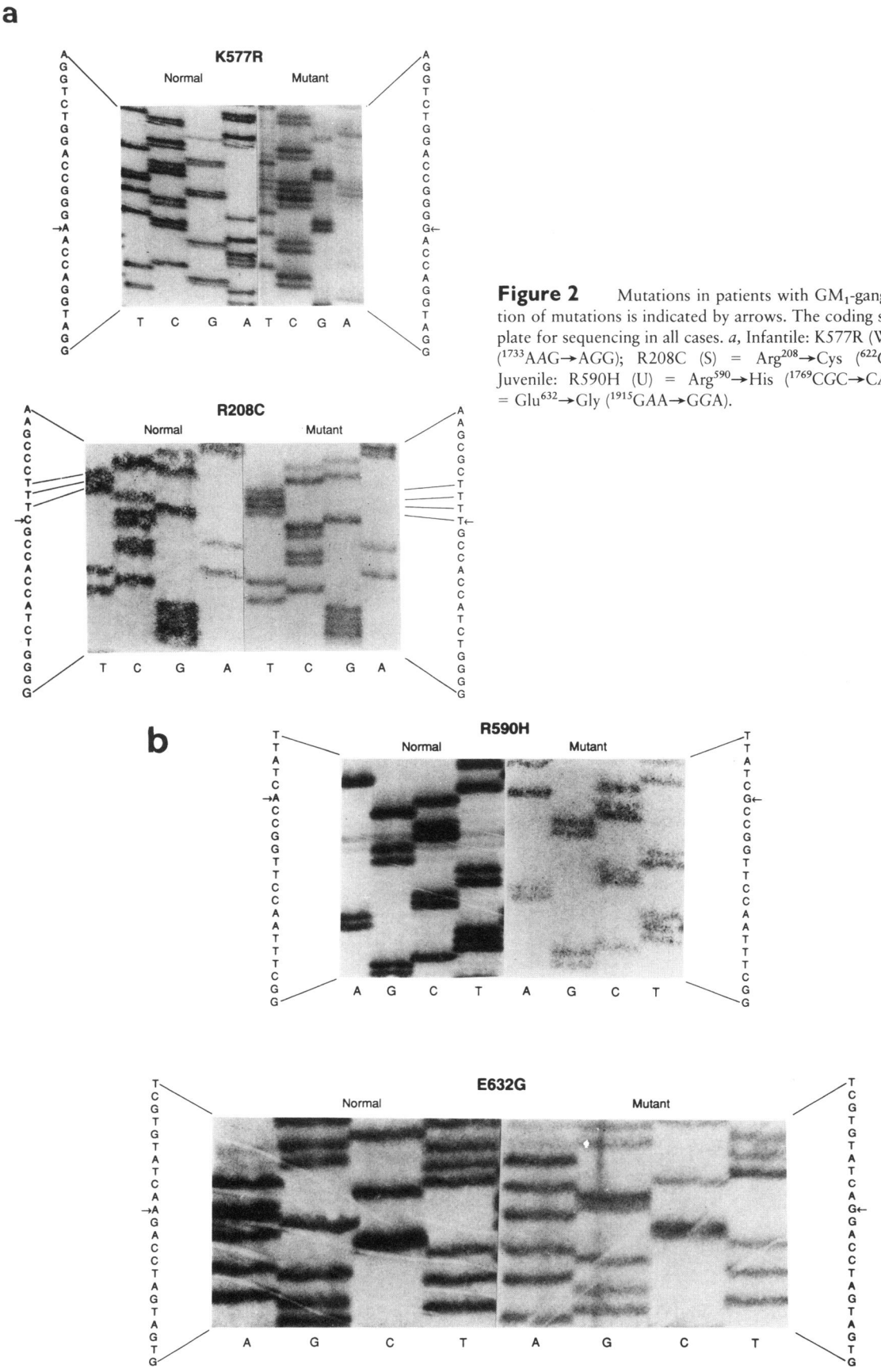

Figure 2 Mutations in patients with GM_1-gangliosidosis. Location of mutations is indicated by arrows. The coding strand is the template for sequencing in all cases. *a*, Infantile: K577R (W) = Lys^{577}→Arg (^{1733}AAG→AGG); R208C (S) = Arg^{208}→Cys (^{622}CGC→*T*GC). *b*, Juvenile: R590H (U) = Arg^{590}→His (^{1769}CGC→CAC); E632G (Z) = Glu^{632}→Gly (^{1915}GAA→GGA).

procedure using the Bio-Rad Protein Assay kit (Bio-Rad Labs, Richmond, CA).

Results

Biochemistry and Northern Blot Analysis

The diagnosis of GM_1-gangliosidosis was confirmed in all patients by deficient activities of acid β-galactosidase in cultured fibroblasts and peripheral leukocytes. All the infantile cases had enzyme activities less than 5% that of normal controls, as did one of the juvenile cases. The second juvenile case had an acid β-galactosidase activity 10% of normal. All cases had normal sialidase activities in cultured fibroblasts (results not shown). Northern blot analysis indicated that both juvenile cases and four infantile cases had an acid β-galactosidase mRNA of normal size and quantity (fig. 1). It was of normal size but reduced in quantity in two infantile cases (cases 4 and 7). A 1.1-kb β-actin cDNA was used as the control transcript to equalize for RNA content.

Sequence Analysis of cDNA

Standard mutation nomenclature is used, but, for purposes of comparison with previously reported Japanese mutations, letters are also assigned. Amplified segments covering the coding sequence were obtained from fibroblasts from all patients. Sequence analysis of the entire coding region revealed four single-base substitutions in the eight patients studied (fig. 2). The most common mutation found was R208C (or S), initially seen in infantile cases 1, 2, 4, and 7. A C→T transition at nucleotide (nt) 622 results in an amino acid change of Arg to Cys at codon 208 in exon 6. A second mutation seen in two infantile cases was K577R (or W), which is a mutation occurring at the next-to-last base at the 3′ end of exon 15. An A→G transition at nt 1733 results in a Lys to Arg substitution at codon 577 in exon 15 (fig. 2*a*). In each of the juvenile cases a different mutation was found, both in exon 16. The first mutation, R590H (or U), was a G→A substitution at nt 1769 resulting in an Arg being replaced by a His at codon 590; the second mutation, E632G (or Z), was an A→G transition at nt 1915 resulting in a Glu to Gly change at codon 632 (fig. 2*b*). In each case multiple mutations were initially found because of PCR artifact. Evaluation of multiple

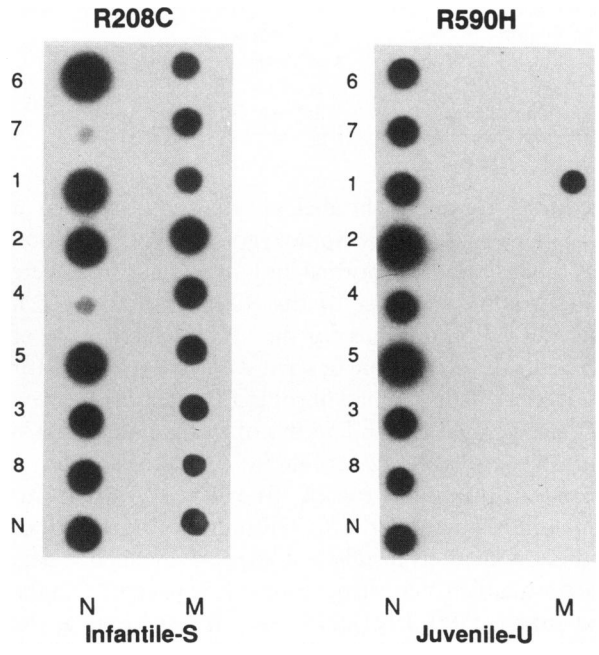

Figure 3 Genotype determination for one of the infantile (R208C) and one of the juvenile (R590H) mutations by allele-specific oligonucleotide hybridization (dot blot). Patients are numbered according to table 1. N = normal probe; M = mutant probe. Infantile cases 4 and 7 are homozygous for the R208C (or S) mutation. Juvenile case 1 is heterozygous with both R590H (U) and R208C (S) alleles.

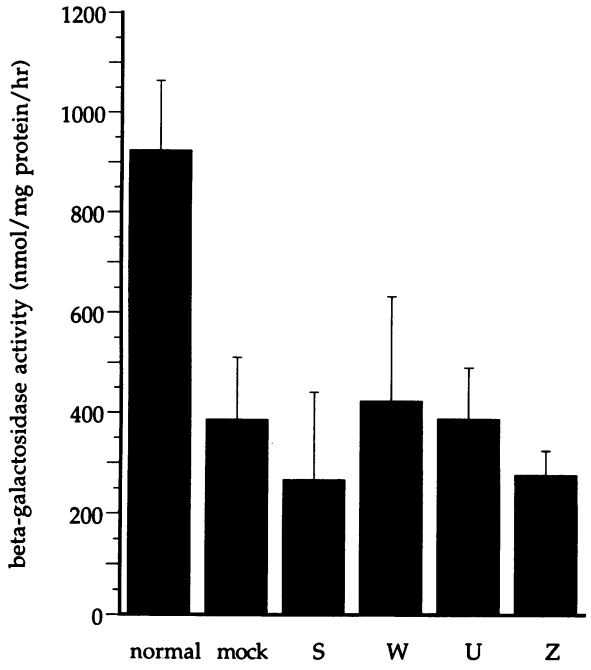

Figure 4 Expression of mutant β-galactosidase in Cos-1 cells. The mutant cDNAs gave catalytic activity toward 4-methylumbelliferyl β-D-galactoside comparable to the intrinsic activity of Cos-1 cells as represented by the mock-transfected cells. The first bar (normal) depicts activities of Cos-1 cells transfected with normal β-galactosidase cDNA. R208C (or S) = Arg^{208}[CGC]→Cys[TGC]; K577R (or W) = Lys^{577}[AAG]→Arg[AGG]; R590H (or U) = Arg^{590}[CGC]→His[CAC]; E632G (or Z) = Glu^{632}[GAA]→Gly[GGA].

Table 2

Human Acid β-Galactosidase Mutations

Author and Nomenclature	Mutant Allele: Type	Mutant Allele: Origin	Mutation
Yoshida et al. (1991):			
I51T (A)	Adult	Japanese	Ile^{51}→Thr
R201C (B)	Juvenile	Japanese	Arg^{201}→Cys
R457G (C)	Adult	Japanese	Arg^{457}→Gln
(D)	Infantile	Japanese	Duplication (nt 1069–1233)
All L10P (E)	Polymorphism	All ethnic groups	Leu^{10}→Pro
Oshima et al. (1992):			
W237L (F)	Morquio B	American	Trp^{237}→Leu
R482H (G)	Morquio B	American	Arg^{482}→His
W509C (H)	Morquio B	American	Trp^{509}→Cys
Yoshida et al. (1991):			
G123R (I)	Infantile	Japanese	Gly^{123}→Arg
Y316C (J)	Infantile	Japanese	Tyr^{316}→Cys
G494C (K)	Infantile	Japanese	Gly^{494}→Cys
(L)	Infantile	Japanese	Duplication (nt 254–276)
Nishimito et al. (1991):			
R49C (P)	Infantile	Japanese	Arg^{49}→Cys
R457Ter (Q)	Infantile	Japanese	Arg^{457}→Ter
Boustany et al. (present study):			
R208C (S)	Infantile	Hispanic	Arg^{208}→Cys
R590H (U)	Juvenile	English	Arg^{590}→His
K577R (W)	Infantile	English	Lys^{577}→Arg
E632G (Z)	Juvenile	English-Scottish	Glu^{632}→Gly
Chakraborty et al. (1991):			
T80M (V)	Adult	Danish-Danish	Thr^{80}→Met

clones (4–6), however, revealed only one of the mutations to be real. Similarly to Nishimoto et al. (1991), some of our patients had a Pro in codon 10 [CCT], while others had a Leu [CTT]. For further verification, mutation-bearing exons were amplified from genomic DNA by PCR and were directly sequenced without cloning into vectors.

Genotype Determination

Amplified genomic DNA was examined for determination of the genotype by means of allele-specific hybridization, using the dot blot method (fig. 3). R208C accounted for one of the alleles in all cases. Also, two of the infantile cases were homozygous for this mutation (see table 1). Twelve normal and disease controls were tested for the presence of the R208C mutation. Ten cases were homozygous for the normal allele. Two of the controls of Hispanic descent were heterozygous for the R208C allele and had fibroblast β-galactosidase levels that were 43.3% and 55.0% of normal (i.e., 44,078 and 56,053 nmol/mg protein/h, respectively, with a mean for normal controls of 101,904 ± 22,770). It was impossible to test for the K577R mutation at the donor end of exon 15, as insufficient intron 15 sequence data were available to construct amplifying primers (Nanba and Suzuki 1990, 1991). However, it was found as the only mutation in the entire coding sequence in two unrelated cases. We, therefore, presume it to be real.

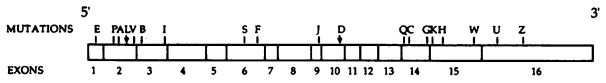

Figure 5 Acid β-galactosidase cDNA. Note two clusterings of mutations at the 5′ end (exons 2 and 3) and 3′ end (exons 14 and 15). Arrows indicate duplications.

Expression Studies in Cos-1 Cells

Cos-1 cells transfected by the pSVL vector carrying acid β-galactosidase cDNA constructs of the four dif-

ferent mutations failed to express β-galactosidase activity over the intrinsic activity. Cos-1 cells transfected with normal β-galactosidase cDNA had 2.5 times the intrinsic activity level (fig. 4). There were no differences observed among the different mutations in multiple independent experiments.

Discussion

These studies identify four new mutations in the acid β-galactosidase gene that were different from the Japanese GM_1-gangliosidosis mutations and from those causing Morquio B (see table 2). Each mutation results in abolishment of the catalytic activity of the enzyme protein as seen in the transient Cos-1 cell expression experiments. Also, all sequenced abnormal alleles contained only one mutation, with the rest of the coding sequence being normal. Hence, each one of the four new mutations described causes GM_1-gangliosidosis. The R208C (S) mutation in exon 6 accounts for 10 of 16 possible alleles. Two infantile cases from Puerto Rico are homozygous for this mutation. The K577R (W) mutation at the next-to-last base of exon 15 accounts for two infantile alleles. Each of R590H (U) or E632G (Z) accounts for the juvenile defining mutation in one of the two juvenile cases. The other allele in both juvenile cases was the infantile R208C allele. But, as noted previously by others (Nishimoto et al. 1991), the mutant enzyme with the higher residual activity will determine clinical phenotype. Although our sample size is limited, none of the juvenile defining alleles (R590H or E632G) was seen in the infantile cases. Two alleles remain unidentified.

Although the R208C mutation was the most common infantile mutation, the presence of at least two others (K577R and unidentified) suggests that mutations underlying infantile GM_1-gangliosidosis in North America and South America are heterogeneous. The common occurrence of the R208C mutation in all cases prompted us to investigate the possibility that it is a commonly occurring mutation in North American and South American populations. Ten normal controls of pure Northern German (5), Swedish-German (1), English-English (3), and English-Scottish (1) descent did not carry the R208C mutation. However, two of our normal controls of Hispanic descent had one allele that expressed the R208C mutation. Those two normal controls had fibroblast β-galactosidase activities in the carrier range. Also, the fact that two GM_1-gangliosidosis cases homozygous for the R208C mutation are of Puerto Rican–Spanish extraction suggests this allele may have spread to North America and South America via Puerto Rico. A larger number of individuals need to be studied before this can be confirmed.

The R208C mutation cannot be a polymorphism, as it abolishes catalytic activity of the mutant protein in Cos-1 cells and was the only identified mutation in the patient-derived full β-galactosidase cDNA. The most compelling reason is that patients who carry this mutation have GM_1-gangliosidosis. Little is known about structure/function relationships of human acid β-galactosidase. However, two clusterings of all described mutations occurring in exons 2 and 3 on the one hand and exons 14 and 15 on the other hand (fig. 5) suggest that these two areas could have important catalytic and/or regulatory functions.

Acknowledgments

The authors wish to thank Drs. G. Thomas and Hugo Moser for supplying us with the GM_1-gangliosidosis cell lines. We thank Mr. Derrick Sauls and Mr. José Langaman for their expert assistance in fibroblast culture. The oligonucleotide probes were synthesized in the Nucleotide Synthesis Laboratory of the program in molecular biology and biotechnology of the University of North Carolina at Chapel Hill and at the Department of Botany DNA synthesis facility at Duke University. This grant was supported in part by research grants HD24061 and NS 25289 and by the Merck Foundation (R.-M.B. is a recipient of the Merck Scholar Award in the neurosciences). Last, but not least, the authors wish to thank Ms. Patsy Martin for her assistance in preparing this manuscript.

References

Chakraborty S, Rafi MA, Wenger DA (1991) A point mutation in the β-galactosidase cDNA sequence of two adult patients with GM_1-gangliosidosis: proceedings of the 8th International Congress of Human Genetics. Am J Hum Genet Suppl 49:A451

Morreau H, Galjart NJ, Gillemans N, Willemsen R, van der Horst GTJ, d'Azzo A (1989) Alternate splicing of β-galactosidase messenger RNA generates the classical lysosomal enzyme and a β-galactosidase-related protein. J Biol Chem 264:20655–20663

Nanba E, Suzuki K (1990) Molecular cloning of mouse acid β-galactosidase cDNA: sequence, expression of catalytic activity and comparison with the human enzyme. Biochem Biophys Res Commun 773:141–148

———(1991) Organization of the mouse β-galactosidase gene. Biochem Biophys Res Commun 178:158–164

Nishimoto J, Nanba E, Inui K, Okada S, Suzuki K (1991) GM1-gangliosidosis (genetic β-galactosidase deficiency): identification of four mutations in different clinical phenotypes among Japanese patients. Am J Hum Genet 49:566–574

O'Brien JS (1989) β-galactosidase deficiency (GM_1-gangliosidosis, galactosialidosis and Morquio syndrome type B); ganglioside sialidase deficiency (mucolipidosis IV). In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic basis of inherited disease. McGraw-Hill, New York, pp 1797–1806

Oshima A, Tsuji A, Nagao Y, Sakuraba H, Suzuki Y (1988) Cloning, sequencing and expression of cDNA for human β-galactosidase. Biochem Biophys Res Commun 157:238–244

Oshima A, Yoshida K, Ishizaki A, Shimmota M, Fukuhara Y, Sakuraba H, Suzuki Y (1992) GM_1-gangliosidosis: tandem duplication within exon 3 of β-galactosidase gene in an infantile patient. Clin Genet 41:235–238

Oshima A, Yoshida K, Shimmoto M, Fukuhara Y, Sakuraba H, Suzuki Y (1991) Human β-galactosidase gene mutations in Morquio B disease. Am J Hum Genet 49:1091–1093

Suzuki K (1987) Enzymatic diagnosis of sphingolipidoses. Methods Enzymol 138:727–762

Wray S, Boustany R-MN (1989) Ophthalmic manifestations of defects in metabolism. In: Tasman W, Jaeger EA (eds) Duane's clinical ophthalmology. Vol 3. JB Lippincott, Philadelphia, pp 1–29

Yoshida K, Oshima A, Shimmota M, Fukuhara Y, Sakuraba H, Yanagisawa N, Suzuki Y (1991) Human β-galactosidase gene mutations in GM_1-gangliosidosis: a common mutation among Japanese adult/chronic cases. Am J Hum Genet 49:435–442