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# GM2-Gangliosidosis B1 Variant: Analysis of β-Hexosaminidase α Gene Abnormalities in Seven Patients

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## Summary

**A single nucleotide transition within exon 5 of the β-hexosaminidase α chain gene was identified in a Puerto Rican patient with GM2-gangliosidosis B1 variant as the mutation responsible for the unusual enzymological characteristics of this variant ($G_{533}$→A; $Arg_{178}$→His) (the DN-allele). A total of seven patients with enzymological characteristics of B1 variant have since been studied. They were Puerto Rican (DN), Italian, French, Spanish, two patients of mixed ethnic origin (English/Italian/Hungarian and English/French/Azores), and a Czechoslovakian. In confirmation of our earlier finding based on screening with allele-specific probes, all patients except the one from Czechoslovakia carried the same DN-allele. A new point mutation found in this patient changed the same codon affected in the DN-allele ($C_{532}$→T; $Arg_{178}$→Cys). An asymptomatic Japanese individual included as a control also carried one allele with the DN-mutation. Site-directed mutagenesis and expression studies in COS I cells demonstrated that either of the two point mutations abolishes the catalytic activity of the α subunit. The Spanish patient was homozygous for the DN-allele, but others were all compound heterozygotes. The Puerto Rican patient was a compound heterozygote with the DN-mutation in one allele and with the four-base insertion in exon 11, one of the two mutations found in the classical Ashkenazi Jewish Tay-Sachs disease, in the other allele. Abnormalities of the other allele were not identified in all other compound heterozygous patients. In these patients, the level of mRNA derived from the other allele was variable, ranging from being undetectable to being much lower than normal. This series of studies uncovered a new B1 variant mutation, confirmed our preliminary finding that the DN-allele has a surprisingly wide geographic and ethnic distribution, and pointed out the highly complex nature of the molecular genetics of this rare disorder. They also support our working hypothesis that mutations responsible for the unique enzymological characteristics of the B1 variant should be located in or near exon 5 of the gene and that this region of the enzyme protein is critical for its catalytic function.**

## Introduction

GM2-gangliosidosis occurs as the result of genetically defective catabolism of a monosialoganglioside, GM2 (Sandhoff et al. 1989). Most forms of the disease are rapidly progressive and fatal, and abnormal accumulation of GM2-ganglioside occurs in neurons throughout the body. Three major categories of GM2-gangliosidosis exist according to the genes that are defective; the α or β subunit of N-acetyl-β-hexosaminidase or the natural activator protein. Tay-Sachs disease, prevalent in the Ashkenazi Jewish population, is the prototype of human GM2-gangliosidosis due to a defective α subunit. Classical Tay-Sachs disease and the phenotypically indistinguishable form occurring in the French-Canadian population produce no or little detectable mRNA or enzyme protein. The French-Canadian vari-

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ant is caused by a major deletion in the 5′ end of the β-hexosaminidase α gene (Myerowitz and Hogikyan 1986, 1987). Almost all patients with classical infantile Ashkenazi Tay-Sachs disease have one of two mutations: (1) a splicing defect at the 5′ donor site of intron 12 and (2) a four-base insertion within exon 11 (Arpaia et al. 1988; Myerowitz 1988; Myerowitz and Costigan 1988; Ohno and Suzuki 1988*a*, 1988*c*). On the other hand, there are variant forms of the disease in which immunologically detectable but catalytically defective enzyme protein is produced. A point mutation in the coding sequence of β-hexosaminidase α gene has recently been identified (Nakano et al. 1988) in a patient in whom the synthesized α subunit protein cannot be transported intracellularly for normal processing ("insoluble α") (Proia and Neufeld 1982). More recently, Navon and Proia (1989) and Paw et al. (1989) identified a point mutation responsible for the adult Ashkenazi Jewish form of the disease. Still unpublished is a transition from G to A at the last nucleotide of exon 5 identified in a Tunisian patient with late-infantile Tay-Sachs disease with less than 10% residual activity of β-hexosaminidase A (L. Poenaru, personal communication). Also among the cross-reactive material-positive forms is the enzymologically unique B1 variant (Goldman et al. 1980; Li et al. 1981; Kytzia et al. 1983). B1 variant patients produce hexosaminidase A (a heterodimer, αβ) which appears normal catalytically when tested with conventional chromogenic or fluorogenic substrates, such as 4-methylumbelliferyl N-acetylglucosaminide, which are split by an active site of the β subunit of the enzyme (Kytzia and Sandhoff 1985). However, patients' hexosaminidase A is catalytically defective against both the natural lipid substrate, GM2, and an artificial substrate, 4-methylumbelliferyl N-acetylglucosamine 6-sulfate. These substrates are hydrolyzed by the active site on the α subunit of normal hexosaminidase A (Kytzia and Sandhoff 1985) which is inactivated in patients' enzyme. Genetic complementation tests confirmed that the B1 variant is allelic to other hexosaminidase α mutations (Sonderfeld et al. 1985). Fibroblasts from patients with this variant synthesize and process an immunologically detectable α subunit of an apparently normal size (Sonderfeld et al. (1985). A specific point mutation in the coding sequence of the β-hexosaminidase α chain was earlier identified in the first described patient with the B1 variant (Ohno and Suzuki 1988*b*). The mutation resulted in an amino acid change from arginine to histidine at residue 178 in the mutant enzyme. Computerized molecular modeling suggested drastic alterations in the three-dimensional structure of the protein in the vicinity of the mutation, consistent with the loss of catalytic activity. In the absence of the standardized nomenclature, this specific mutation (a single nucleotide transition from G to A at 533) will be referred to in this report as the *DN-allele.* Ohno and Suzuki (1988*b*) hypothesized that this region of the enzyme protein is important in defining its catalytic activity and that patients with the unique enzymological characteristics of the B1 variant might have abnormalities in the same region, even though the mutations may be different in different patients. A preliminary study of six patients by allele-specific synthetic oligonucleotide probes indicated a surprisingly wide geographic and ethnic distribution of the DN-allele (Tanaka et al. 1988). The present report describes results of studies on a total of seven B1 variant patients, which demonstrated that (1) a new point mutation, affecting the same codon, is responsible for the disease in a Czechoslovakian patient; (2) either of the point mutations inactivates the catalytic activity of the enzyme; (3) except for one Spanish patient, all patients were compound heterozygotes; (4) the other allele in the original Puerto Rican patient was one of the infantile Jewish Tay-Sachs alleles, but other compound heterozygous patients had neither of the infantile Jewish alleles; and (5) the other alleles were mRNA positive in some patients and mRNA negative in others.

## Material and Methods

### *Patients*

Altogether, seven patients diagnosed enzymatically as having the GM2-gangliosidosis B1 variant were included in the present study: our original Puerto Rican patient (Goldman et al. 1980); one patient each from Italy, France, Spain, and Czechoslovakia; and two patients of mixed ethnic background (the maternal grandparents of one patient were English-Scottish and German-English, and the paternal grandparents were Italian and Hungarian, respectively; and the other patient was of English/French background with grandparents of both sides from the Azores). The patient from France was included in one of our earlier reports (Kytzia et al. 1983), and the one from Czechoslovakia is also in the literature (Conzelmann et al. 1985). The pertinent clinical and enzymological information on these patients is given in table 1. Our preliminary study included all of these patients except case 7 (Tanaka et al. 1988).

**Table 1**

**Summary of Clinical and Enzymological Information**

| Case | Sex | Ethnic Background | Age | | β-Hexosaminidase Activity[a] (nmol/min/mg protein) | | |
|---|---|---|---|---|---|---|---|
| | | | Onset | Death | 4-MU GlcNAc Total Activity | %A | Sulfated Substrate |
| 1[b] .... | M | French | 2 years, 1 mo | 7 years, 1 mo | 107 | 50% | .21 |
| 2[c] .... | M | Czechoslovakia | 8 mo | 1 year, 11 mo | 101 | 30% | .20 |
| 3 .... | F | Italian | "Juvenile" | | 71.4 | 72% | .13 |
| 4 .... | F | Spanish[d] | 2–3 years | 13 years, 2 mo | 96.3 | 61% | .22 |
| 5[e] .... | M | Puerto Rican | 11 mo | 4 years, 8 mo | 132 | 51% | .31 |
| 6 .... | M | English/Italian/Hungarian | 2–3 years | Still alive | 90.9 | 62% | .19 |
| 7 .... | M | English/French/Azores[f] | 4 years | Still alive | 90.5 | 49% | .04 |

Note.—There was no positive record of consanguinity for any of the patients.

[a] Normal control activities ($n$ = 7) for the nonsulfated and sulfated substrates were 154 ± 21.4 (A = 65 ± 11%) and 18.7 ± 6.5, respectively.

[b] Reference: Kytzia et al. (1983).

[c] Reference: Conzelmann et al. (1985).

[d] Parents were from the same village.

[e] References: Goldman et al. (1980), Kytzia et al (1983), and Ohno and Suzuki (1988*c*).

[f] Grandparents of both sides were from the Azores.

### *Commercial Materials*

Bethesda Research Laboratories (Gaithersburg, MD), Boerhinger Mannheim (Indianapolis), Pharmacia (Piscataway, NJ), International Biotechnologies Inc. (New Haven, CT), and New England Biolab (Beverley, MA) were the main sources for enzymes, reagents, and other molecular biological supplies. Radioisotopes were obtained from ICN Radiochemicals (Irvine, CA). 4-Methylumbelliferyl β-N-acetylglucosaminide was purchased from Sigma Chemical Co. (St. Louis), and 4-methylumbelliferyl β-N-acetylglucosamine 6-sulfate was from the HSC Research Development Corp. (Toronto). Transformed African green monkey kidney cells (COS I) were purchased from the American Type Culture Collection (Rockville, MD). Sources for nonstandard materials will be indicated below as appropriate.

### *Fibroblast Cultures*

For all patients, cultured fibroblasts were used as the source materials for the study. Besides our original patient, individual cell lines were shipped to Chapel Hill, NC, from Boston, Bonn, and Lyon. They were maintained and propagated under the standard conditions of the laboratory for a minimum of a few weeks before harvesting.

### *Enzymatic Assays*

β-Hexosaminidase A and B activities were determined on total homogenates of cultured fibroblasts. 4-Methylumbelliferyl β-N-acetylglucosaminide was used as substrate for differential assays for the A and B isozymes by the heat inactivation method, and 4-methylumbelliferyl β-N-acetylglucosamine 6-sulfate was used for direct assessment of the activity of the A isozyme (Suzuki 1987). The amount of protein was determined essentially by the method of Lowry et al. (1951) with BSA as the standard.

### *DNA Polymerase Chain-Reaction and DNA Sequence Analysis*

Genomic DNA was isolated according to the standard procedure (Maniatis et al. 1982). Two 20-mer primers were synthesized for the polymerase chain-reaction procedure for amplification of genomic sequences (Kogan et al. 1987; Wong et al. 1987; Oste 1988; Stoflet et al. 1988). One of them was within intron 3, approximately 85 bases upstream of exon 4 (5′-GCTCTGCTACATTGAGAACC), and the other was within intron 5, about 30 bases downstream of exon 5 (5′-CTGTCCGTTGCTCCATCACC). Since intron 4 is approximately 0.5 kb, the distance between the two

primers in the gene was about 800 bp. The protocol for the reaction was as follows: The target sequence was amplified in a 100-μl reaction vol containing 5 μg genomic DNA in 50 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.2 mM each of dNTP, and 1 μM of each primer in a siliconized microcentrifuge tube. Samples were heated at 94°C for 6 min, centrifuged momentarily, and annealed at 55°C for 3 min. Two and a half units of *Taq* polymerase was added, and the samples were incubated at 72°C for 3.5 min for extension with an overlayer of 100 μl mineral oil. The manual cycling profile was 55°C for 2.5 min (annealing), 72°C for 3.5 min (extension), and 94°C for 1.5 min (denaturation). Usually, the extension reaction was repeated 40 times. *Taq* polymerase (2.5 units) and the primers (30 pmol each) were added at the 20th cycle, and *Taq* polymerase only (2.5 units) was added at the 30th cycle. The procedure was terminated after the 41st extension reaction, which was run for 7 min. In later experiments, an automated thermal cycler apparatus was used for the polymerase chain-reaction, instead of the manual procedure described above. In the automated procedure, the cycling times for annealing, extension, and denaturation were 2, 3, and 1 min, respectively, and 3.5 units of *Taq* polymerase was added only at the beginning of the cycling procedure. *Taq* polymerase was purchased from Cetus-Perkin Elmer Corp. (Norwalk, CT). After amplification, a portion of the reaction mixture was subjected to agarose-gel electrophoresis to ascertain presence of the amplified 800-bp sequence. The remainder was digested with *Bam*HI and *Stu*I. A *Bam*HI site is located in intron 4, and the *Stu*I site is within intron 5 near its 5′ end. Thus, the digestion generated a *Bam*HI-*Stu*I fragment that included most of the intron 4 sequence, the entire exon 5, and a short segment of intron 5. The fragment was subcloned into appropriate M13 vectors, and the sequence analysis was carried out by the Sanger dideoxy chain-termination method (Sanger et al. 1977) with the 17-mer sequencing primer and $^{35}S$-labeled dATP (Biggin et al. 1983). The DNA polymerase used for sequencing was either the Klenow enzyme or the T7 polymerase (Sequenase; US Biochemical Corp., Cleveland).

### *Expression of the Catalytic Activity in the COS I System*

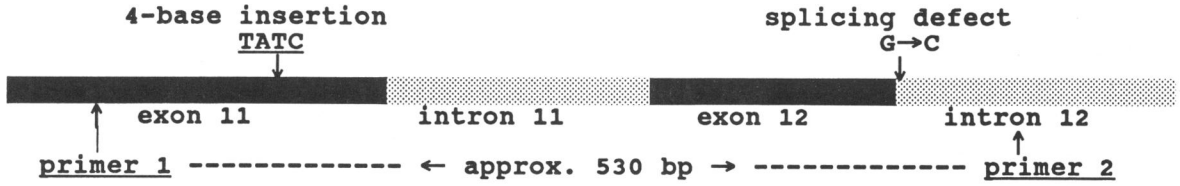
The catalytic activity of the enzyme protein produced by the β-hexosaminidase α cDNA clone was expressed in the COS I cell system. The normal hexosaminidase α cDNA clone (Myerowitz et al. 1985) was digested with *Nar*I to leave only 60 bases of 5′-untranslated sequence above the initiation codon and was subcloned into a eukaryotic expression vector, pSVL. This was the same strategy used recently by Navon and Proia (1989). The COS I cells were transfected by the DEAE-dextran-mediated procedure (Cullen 1987). β-Hexosaminidase activities were assayed 48 h after transfection with 4-methylumbelliferyl β-N-acetylglucosaminide and 4-methylumbelliferyl β-N-acetylglucosamine 6-sulfate, as described above. For expression of the clones with the DN mutation, both the native full-length mutant clone obtained from the patient's fibroblasts and the clone generated from the normal cDNA by site-directed mutagenesis were examined. For the new B1 variant mutation, only the mutation generated in the normal cDNA by site-directed mutagenesis was examined, because native full-length mutant cDNA was not available. Entire experiments were repeated at least once more and often a few times, with duplicate plates in each experiment to insure reproducibility of the results.

### *Site-directed Mutagenesis*

Either of the B1 variant mutations ($G_{533}\rightarrow A$ or $C_{532}\rightarrow T$) were introduced into the normal β-hexosaminidase α cDNA clone by site-directed mutagenesis using a commercially available mutagenesis kit (Muta-Gene Phargemid In Vitro Mutagenesis Kit; Bio-Rad Labs, Richmond, CA) according to the procedure provided by the manufacturer. The vector was a phargemid, $pTZ18U^{amp+}$, and the host *E. coli* were $CC2MV1190^{dut+,ung+,cp-,amp-}$ and $CJ236^{dut-,ung-,cp+,amp-}$. The transformants were analyzed by sequencing with an antisense 15-mer primer that hybridized at 15 bases downstream of the mutagenized nucleotide (5′-GTCCAGGATGCTAGAGAG). The efficiency of the mutagenesis was approximately 75%. Correctly mutagenized clones were subcloned into pSVL for subsequent expression experiments in the COS I cells as above.

### *Screening for the Infantile Ashkenazi Jewish Alleles with Single Polymerase Chain-Reaction and Allele-specific Probes*

The strategy is depicted in figure 1. Two primers were used to amplify a segment of β-hexosaminidase α chain gene that included both the regions of the four-base insertion in exon 11 and the exon 12–intron 12 junctional mutation found in the classical infantile Ashkenazi Jewish Tay-Sachs disease. The 5′ primer was within exon 11 at the same segment as used by Myerowitz and Costigan (1988) (5′-GTGTGGCGAGAGGATATTCCA). The 3′ primer was from the intron 12 sequence, approximately 80 bases downstream of the 5′ donor site (Ohno and Suzuki 1988*a*) (5′-AGACCC-




Primer 1: 5'-GTGTGGCGAGAGGATATTCCA (exon 11)
Primer 2: 5'-AGACCCAATCCCATCTAGCCA (intron 12)

Probes: 5'-GAACCGTATATCCTATGGC (insertion, normal)
5'-GAACCGTATATCTATCCTA (insertion mutant)
5'-CCAGGCTCTGGTAAGGGTTTT (splicing defect, normal)
5'-CCAGGCTCTGCTAAGGGTTTT (splicing defect, mutant)

**Figure 1** Strategy for screening for the two classical infantile Ashkenazi Jewish Tay-Sachs disease alleles. A single polymerase chain reaction amplifies the segment of the gene that includes both sites for the two mutations known to occur in the infantile Ashkenazi disease. Then two pairs of allele-specific probes are used to detect the normal and mutant sequences by dot hybridization.

AATCCCATCTAGCCA). The amplified segment was approximately 530 bp, including two-thirds of exon 11, all of both intron 11 and exon 12, and about 80 bases of intron 12. Two pairs of allele-specific oligonucleotide probes were synthesized, one pair of normal and mutant sequences for the four-base insertion and one pair of normal and mutant sequences for the splicing-defect mutations: insertion, 5′-GAACCGTATATCCTATGGC (normal) and 5′-GAACCGTATATC TATCCTA (mutant); splicing defect, 5′-CCAGGCTCTGGTAAGGGTTTT (normal) and 5′-CCAGGCTCTGCTAAGGGTTTT (mutant).

The amplified sequence was examined by agarose-gel electrophoresis, and the quantity was estimated. Approximately 10 ng amplified products was applied to Gene Screen Plus membrane (NEN Research Products, Boston) and dried. The prehybridization was in 6 × SSC, 5 × Denhardt's solution, 0.05% sodium pyrophosphate, 0.5% SDS, and 100 μg heat-denatured salmon-sperm DNA/ml at 37°C for 1 h. Hybridization was in the same mixture, except for 1 × Denhardt's solution without SDS and the addition of 50 pmol probes labeled at the 5′ end with [γ-$^{32}$P]ATP and polynucleotide kinase. The specific activity of the probes was generally in the range of 1.5–2.0 × $10^4$ cpm/pmol. Hybridization was continued overnight with gentle shaking, at 44°C for the probes for the splicing defect and at 37°C for the probes for the four-base insertion. These temperatures were chosen as $[T_d-(16–20)]$°C. The membranes were washed in 6 × SSC, 0.05% sodium pyrophosphate four times for 10 min each at room temperature and then were washed twice more for 15 min each in the same solution but at $[T_d-5]$°C. The dried membrane was exposed to X-ray film at −70°C with the Cronex intensifying screen.

### *Screening for Stable mRNA Derived from the Non-B1 Allele*

Since six of the seven B1 variant patients examined were compound heterozygotes and since five did not possess either of the infantile Ashkenazi Jewish alleles, attempts were made to detect mRNA molecules which do not contain the B1 variant sequences. Total RNA was isolated, by the standard procedure (Ausubel et al. 1989), from cultured fibroblasts. Some of the normal control RNA was isolated from leukocytes prepared from freshly drawn blood. The polymerase chain-reaction was carried out with two primers that were complementary to normal mRNA segments flanking the exon 5 sequence: 5′-GTCCAGGATGCTAGAGAG and 5′-TCCGAGAGGGGAGACCAGCGG. The first reaction was with the downstream primer and avian myeloblastosis virus (AMV) reverse transcriptase (Promega Corp., Madison, WI), according to the method of Sarkar and Sommer (1988) but with additional 4 mM sodium pyrophosphate and 1 unit RNAsin/μl in the reaction mixture. Then the remainder of the amplification procedure was essentially the same as de-

scribed above. Three 21-mer oligonucleotides all centered around nucleotide 533 were used as allele-specific probes: the normal sequence (nucleotide 532 = C; nucleotide 533 = G), the DN sequence (nucleotide 533 = A), and the new B1 variant sequence (nucleotide 532 = T). Dot blotting, hybridization, and autoradiography were done in manners essentially similar to those described above for the genomic DNA screening.

## Results

The results of the polymerase chain-reaction and subsequent sequencing of the amplified gene segments were consistent with the analyses with the oligonucleotide probes (Tanaka et al. 1988). The complete sequence of exon 5 and the flanking sequences of intron 4 and intron 5 could be determined by this procedure. In all patients, at least six—and, in some cases, as many as a dozen—subcloned fragments were sequenced for statistical reliability. In addition, whenever a sequence which was neither normal nor of the DN-allele was encountered, the entire procedure, from the amplification of genomic DNA through sequencing, was repeated in order to exclude possible artifacts due to polymerase errors. As to be discussed later, this precaution proved to be critical. Two different mutations were identified within exon 5 (figs. 2 and 3). The DN-allele was present in six patients. The only exception was the patient from Czechoslovakia, who had the new B1 variant allele. The mutation in this patient was a single nucleotide transition in the same codon as in the DN-allele. The transition in this case was in nucleotide 532, from the normal C to T in the mutant. This mutation in the first base of the codon results in a point mutation of amino acid 178, from arginine to cysteine, in contrast to the DN-mutation, which changes the same arginine to histidine because of the transition of the second nucleotide of the codon.

The normal exon 5 and flanking sequence were also detected in all patients except in the Spanish patient (case 4), indicating frequent compound heterozygosity, as expected for this rare genetic condition. Only the DN-allele sequence was found in the Spanish patient. When this fact is considered together with the finding with the oligonucleotide probes (Tanaka et al. 1988), it is likely that this patient was homozygous for the DN-allele.

The results of expression of the catalytic activity in the COS I cells are summarized in figure 4. Transfection with the normal β-hexosaminidase α clone resulted in twofold and six- to sevenfold increases of the baseline activity with the nonsulfated and sulfated substrates, respectively. These findings are consistent with β-hexosaminidase S (αα) being primarily responsible for the increase in the catalytic activity. Excess production of the α subunit in the system is expected to generate excess of the S isozyme, which has very low catalytic activity toward the nonsulfated substrate but is active to-

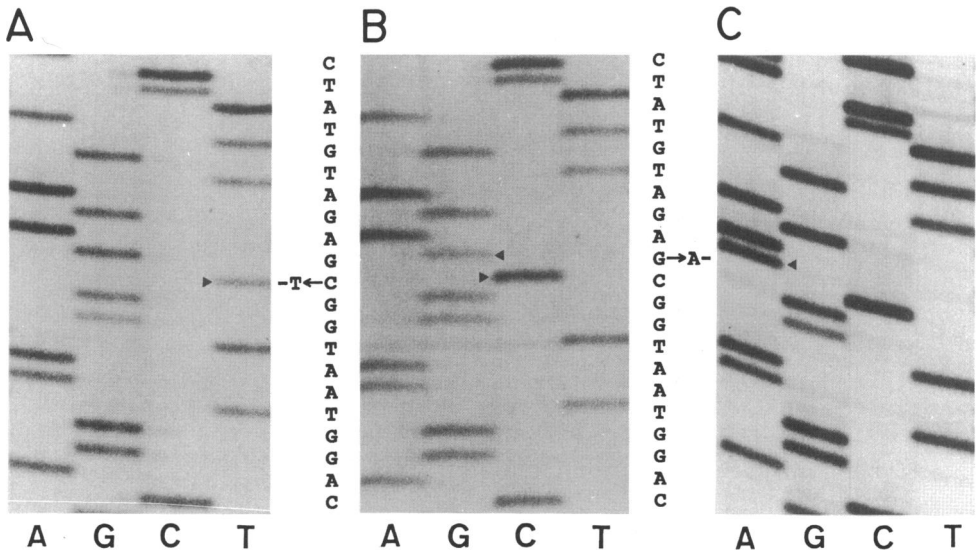



**Figure 2** Mutations in GM2-gangliosidosis B1 variant: sequence analysis of exon 5 of the amplified β-hexosaminidase α gene. *A*, DN-allele. *B*, normal gene sequence. *C*, Sequence in the Czechoslovakian patient. The sequences represent the noncoding strand, with the bottom of the gel being the 5′ side. The area shown corresponds to nucleotides 523–543 in fig. 3.

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          exon 4 ¦ exon 5
451   GAG GGC ACA[TTC TTT ATC AAC AAG ACT GAG ATT GAG GAC TTT CCC
151   Glu Gly Thr[Phe Phe Ile Asn Lys Thr Glu Ile Glu Asp Phe Pro

                                                    TGC
                                                    CAC
496   CGC TTT CCT CAC CGG GGC TTG CTG TTG GAT ACA TCT CGC CAT TAC
166   Arg Phe Pro His Arg Gly Leu Leu Leu Asp Thr Ser Arg His Tyr
                                                    His
                                                    Cys

541   CTG CCA CTC TCT AGC ATC CTG GAC ACT CTG]GAT GTC ATG GCG TAC
181   Leu Pro Leu Ser Ser Ile Leu Asp Thr Leu]Asp Val MET Ala Tyr
                                    exon 5 ¦ exon 6
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**Figure 3** Exon 5 and flanking regions of exons 4 and 6 sequences of β-hexosaminidase α gene, showing the location of the two mutations responsible for the B1 variant phenotype. The boundaries of exon 5 and introns are indicated by square brackets.

ward the sulfated substrate. The clones with either of the B1 variant mutations, natural or generated by mutagenesis, completely failed, with either of the substrates, to increase the catalytic activities above the baseline.

Amplification of a single genomic segment containing both regions of the two mutations responsible for the classical infantile Ashkenazi Jewish Tay-Sachs disease and subsequent screening with oligonucleotides specific for the respective alleles provided a rapid and reliable procedure for detection of these mutant genes

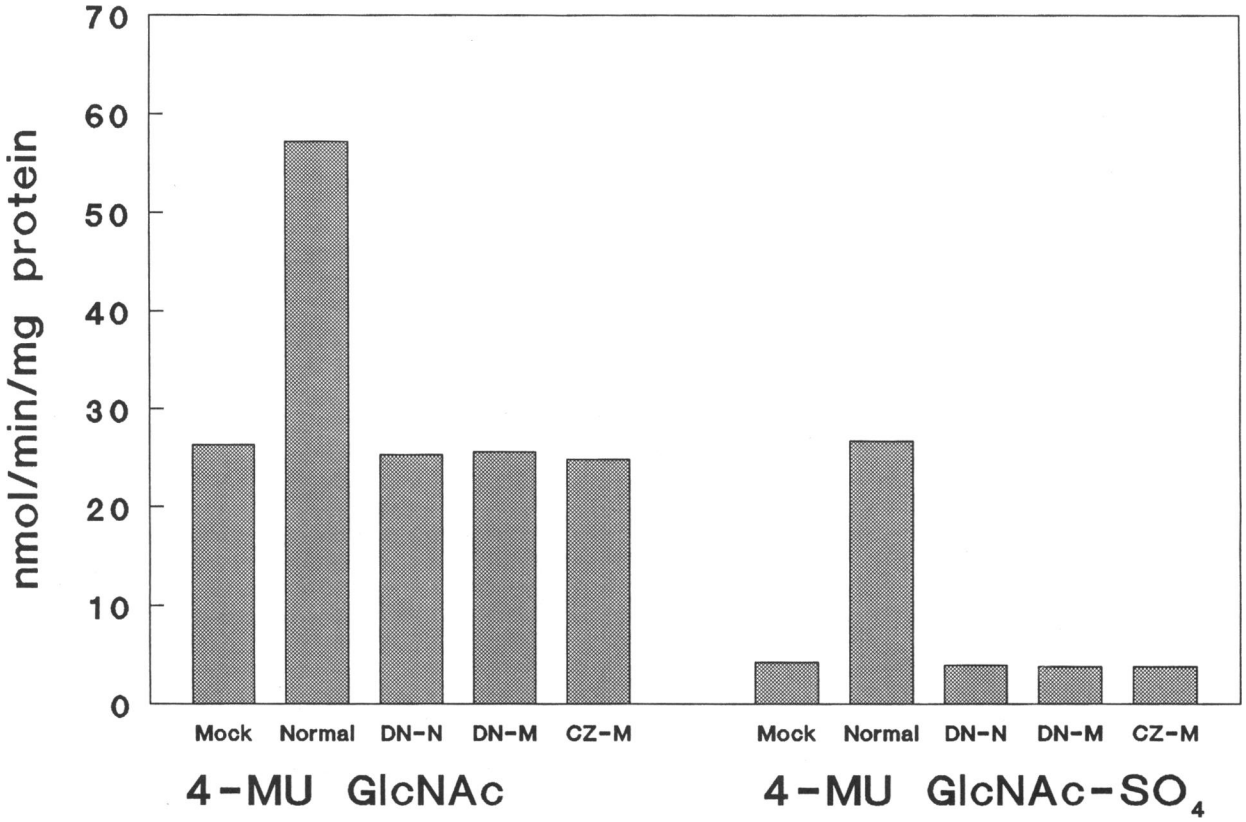


**Figure 4** Expression of the catalytic activity in COS I cells. The methodological details are described in the text. DN-N = DN-mutant cDNA isolated from the patient; DN-M and CZ-M = mutant cDNAs corresponding to the DN-mutation and to that in the Czechoslovakian patient, respectively, and generated from the normal cDNA by site-directed mutagenesis.

(fig. 5). None of the six compound heterozygous patients had the gene with the splicing defect. However, our original Puerto Rican patient was heterozygous with the four-base insertion. Thus, except for both the Spanish patient (case 4), who is likely to be a B1 homozygote, and the Puerto Rican patient (case 5), the abnormalities of the other allele in these patients remain unidentified. Incidental but informative observations were made regarding specimens from patients with various clinical phenotypes of Tay-Sachs disease who were included in the experiment (fig. 5). Three patients with infantile Tay-Sachs disease—Japanese, a black, and a "Caucasian"—had neither of the Jewish alleles. One 3-year-old patient recorded as "Caucasian" (GM0221) was homozygous for the four-base insertion. Of the three Jewish patients, GM502B was a compound heterozygote with both the splicing defect and the four-base insertion, confirming the previous results (Myerowitz 1988; Myerowitz and Costigan 1988; Ohno and Suzuki 1988*a*, 1988*c*). The other two Jewish individuals, GM0515A and GM3052, both carried the four-base insertion in one allele but no splicing defect in the other allele. GM3052 is an obligate heterozygous carrier of a Jewish patient known to carry both of the abnormal alleles. In agreement with Myerowitz and Costigan (1988), the results on GM0515A indicate that this 1-year-old Jewish patient carries the four-base insertion in one allele and another, yet to be identified abnormality in the other. While many of these observations are confirmatory of previous results, they demonstrate usefulness of both the single amplification and the subsequent screening with the allele-specific probes as a rapid diagnostic procedure.

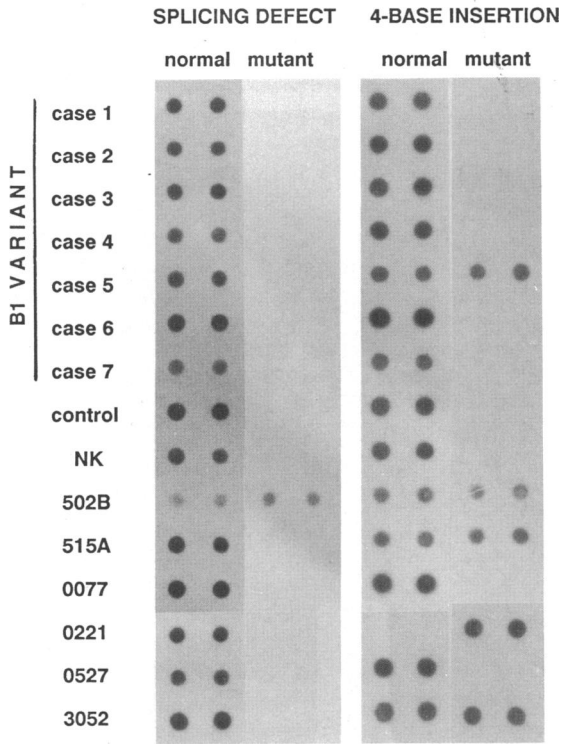


**Figure 5** Screening for the two classical infantile Ashkenazi Jewish Tay-Sachs disease alleles. The strategy is depicted in fig. 1. Methodological details are described in the text. Each sample was dot-blotted in duplicate. Among the seven B1 variant patients, only case 5 (Puerto Rican) has the four-base insertion in the other allele. Identification of other samples are as follows: NK = a Japanese, 16 mo; 502B = Ashkenazi Jewish, 11 mo; 515A = Ashkenazi Jewish, 1 year; 0077 = a black, 1 year; 0221 = "Caucasian," 3 years, 0527 = "Caucasian," 15 mo; 3052 = Ashkenazi Jewish, 24 years, obligate heterozygous carrier. The figure was prepared from a single X-ray film, although for a clearer presentation samples were rearranged from the original 12 samples/row.

Since the screening of the amplified genomic sequence showed five of the seven patients to have unidentified abnormality in the other allele, attempts were made to detect mRNA that has the normal sequence in the region of the B1 mutations. A positive signal would indicate presence of mRNA derived from the other allele. The results were complex (fig. 6). Relatively even density for the mutant probes for all samples assured that semiquantitative comparison was possible among the samples for the normal sequence. As expected, mRNA with the normal sequence around nucleotides 532 and 533 was not detected either in the Spanish patient, who is homozygous for the DN-allele, or in the Puerto Rican patient, who has the four-base insertion in the other allele, which is known to be essentially mRNA negative. In addition to these two cases, no signal was visible for the normal sequence in the French patient (case 1). Very faint but visible signals were obtained for cases 2 and 6. The signals for cases 3 and 7 were moderately strong but still much less than those of the normal control, even after the double gene dosage in the normal control is considered. Unexpectedly, an asymptomatic Japanese individual included as a control appeared to carry the DN-mutation in one allele, further indicating the widespread nature of this particular mutation (fig. 6).

## Discussion

When we identified the point mutation in our original Puerto Rican patient with the GM2-gangliosidosis B1 variant, we postulated that, in view of the rather unique enzymological characteristics of the variant, mutations responsible for the disease in other B1 variant

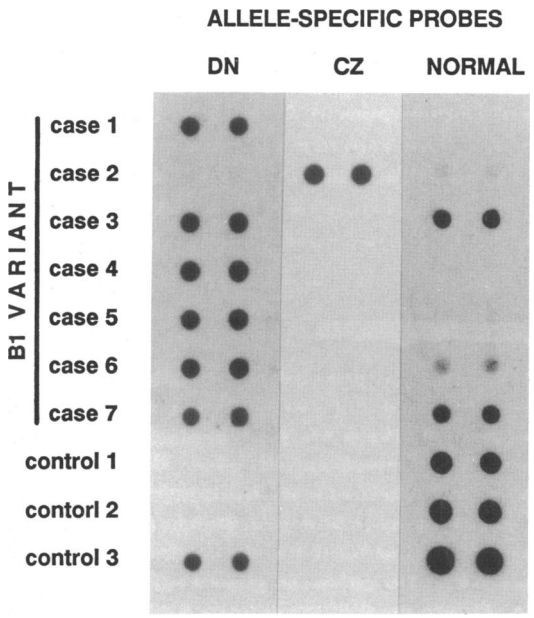


**Figure 6** Levels of detectable β-hexosaminidase α chain mRNA. Methodological details are described in the text. Each sample was dot-blotted in duplicate. All B1 variant patients produce mRNA with either of the mutations identified for the variant (DN = $G_{533}$ to A; CZ = $C_{532}$ to T). As expected, no mRNA with the normal sequence in this segment is detectable in case 4 (homozygous for the DN-allele) and case 5 (the other allele has the Ashkenazi four-base insertion, known to be mRNA negative). Although much less than normal, mRNA from the other allele is clearly present at significant amounts in cases 3 and 7 and is barely detectable in cases 2 and 6. mRNA from the other allele could not be detected in case 1. Control 3 is an asymptomatic Japanese individual who appears to carry one DN-allele.

patients might be in the same region of the enzyme protein (Ohno and Suzuki 1988*b*). We anticipated, however, that the specific mutations in individual patients will probably be different, because the disease is very rare and is distributed widely in different ethnic groups in the world. The findings on the Czechoslovakian patient support our hypothesis. This patient had a new point mutation not just within exon 5 but in fact in the same codon. As a result, $Arg_{178}$ in normal enzyme protein is changed to cysteine in this case instead of to histidine as in the DN-allele. Experiments with site-directed mutagenesis and expression in the COS I cells showed that either of these mutations abolishes the catalytic activity. Thus, $Arg_{178}$ must be critical for the catalytic activity of the enzyme. On the other hand, the enzymological characteristic of the Czechoslovakian patient was different from that of the other patients in that β-hexosaminidase A activity toward 4-methylumbelliferyl β-glucosaminide was partially deficient, while it was essentially normal in other patients (table 1).

The wide geographic and ethnic distribution of the DN-allele was first indicated by allele-specific oligonucleotide screening (Tanaka et al. 1988) and has now been confirmed by actual sequencing. The same DN-mutation is responsible for six of the seven B1 variant patients so far examined, the exception being the Czechoslovakian patient. Furthermore, the DN-mutation was also found in an asymptomatic Japanese individual by the oligonucleotide screening. This finding was surprising and was somewhat contrary to our prediction, even though G→A/C→T transitions are much more common than transversions. Although three patients might be considered as having a Hispanic background (patients 4, 5, and 7), a common origin of these individuals is not obvious.

Only in two of the seven patients examined could the gene abnormalities in both alleles be identified. The Spanish patient is the only known B1 variant patient to date who is homozygous for the DN-allele. This is consistent with the family history that the parents of this patient are from the same village in Spain, although there is no positive record of consanguinity. The Puerto Rican patient was a compound heterozygote of the DN-allele and the four-base insertion of the infantile Ashkenazi Jewish allele. Even in the other compound heterozygous individuals, the other allele is a priori abnormal, since they would not otherwise have been symptomatic. Whatever the nature of the abnormality in the other allele might be, we found at least exon 5 and the flanking intron sequences to be normal in these patients. If the other allele is of both mRNA-positive and cross-reacting material-positive type, a mixture of two abnormal α chains will be generated, possibly resulting in complex enzymological characteristics. If, on the other hand, it is of the mRNA-negative type, there will be only one type of enzyme protein derived from the B1 variant alleles. The enzymological characteristics of these patients were generally typical of the B1 variant—normal or moderately low hexosaminidase A activity toward the unsulfated fluorogenic substrate and disproportionately defective activity toward GM2-ganglioside and/or the sulfated artificial substrate. Three possibilities can account for these findings: (1) the other allele may not be producing any stable enzyme protein; (2) the protein product of the other allele is totally inactive catalytically, or (3) mutations outside exon 5 region can produce a mutant protein that has enzymological characteristics indistinguishable from that of the B1 variant. The levels of mRNA derived from the other

allele were so low in cases 1, 2, and 6 that the quantity of the enzyme protein would be probably insignificant even if it is stable. Only in cases 3 and 7 were there sufficiently high levels of mRNA from the other allele to potentially result in a significant quantity of another mutant enzyme protein, if it is stable. these considerations clearly point to the genetic complexities and the intrinsic limitation of the enzymological classification of this class of genetic disorders.

Our original B1 patient was first diagnosed as an atypical case of AB variant (GM2 activator deficiency) because of the normal β-hexosaminidase A and B activities with the fluorogenic substrate (Goldman et al. 1980). One of the atypical features was the slightly later onset and slower progression of the disease compared with those of patients having typical infantile Tay-Sachs disease. From the limited number of patients examined in the present study, B1 variant patients with the DN-allele ($Arg_{178}$→His) are generally in the late-infantile to juvenile age groups (table 1). The Czechoslovakian patient who has the new B1 variant mutation ($Arg_{178}$→Cys), however, had the clinical onset and course more typical of a patient having infantile Tay-Sachs disease. As more B1 variant mutations are expected in the future, clinical and enzymological features of the B1 variant will certainly be complex, and no generalization is possible. For example, some of the patients in the literature who have been described as having juvenile GM2-gangliosidosis with partial deficiency of β-hexosaminidase A may well have had mutations in the same region of the β-hexosaminidase α chain (Suzuki et al. 1970; Suzuki and Suzuki 1970; Zerfowski and Sandhoff 1974). Previously, B1 variant was thought to occur when a mutation in the α subunit gene abolishes its catalytic activity toward the nonsulfated substrate but retains the activity toward both the natural substrate, GM2-ganglioside, and the sulfated substrate. This notion is not quite correct conceptually. In view of the different nature of the active sites on the α and β subunits (Kytzia and Sandhoff 1985), the enzymological characteristics of the B1 variant would be expected whenever a mutation in the α subunit results in a stable but catalytically inactive α chain that is still capable of normally associating with the β subunit to form the A isozyme. Under such circumstances, the activity toward 4-methylumbelliferyl β-glucosaminide will be normal because the A isozyme includes the normal catalytic site on the β subunit.

During the course of the polymerase chain-reaction and sequencing, we encountered a few artifactual "mutations." Two of them were found in multiple clones after cloning of the amplified sequence into the M13 vector. Therefore, whenever we found abnormal sequences, the entire procedure was repeated, from the step of amplification of the genomic DNA through sequencing. Our experience indicates that this precaution is critical in order to exclude abnormalities due to polymerase errors, when amplified segments are cloned into vectors for subsequent sequencing.

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