

Genetic heterogeneity in type 1 Gaucher disease: Multiple genotypes in Ashkenazic and non-Ashkenazic individuals

(lysosomal/sphingolipidosis/glucocerebrosidase)

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ABSTRACT Nucleotide sequence analysis of a genomic clone from an Ashkenazic Jewish patient with type 1 Gaucher disease revealed a single-base mutation (adenosine to guanosine transition) in exon 9 of the glucocerebrosidase gene. This change results in the amino acid substitution of serine for asparagine. Transient expression studies following oligonucleotide-directed mutagenesis of the normal cDNA confirmed that the mutation results in loss of glucocerebrosidase activity. Allele-specific hybridization with oligonucleotide probes demonstrated that this mutation was found exclusively in the type 1 phenotype. None of the 6 type 2 patients, 11 type 3 patients, or 12 normal controls had this allele. In contrast, 15 of 24 type 1 patients had one allele with this mutation, and 3 others were homozygous for the mutation. Furthermore, some of the Ashkenazic Jewish type 1 patients had only one allele with this mutation, suggesting that even in this population there is allelic heterozygosity. These findings indicate that there are multiple allelic mutations responsible for type 1 Gaucher disease in both the Jewish and non-Jewish populations. Allelic-specific hybridization demonstrating this mutation in exon 9, used in conjunction with the *Nci* I restriction fragment length polymorphism described as a marker for neuronopathic Gaucher disease, provides a tool for diagnosis and genetic counseling that is ~80% informative in all Gaucher patients studied.

Gaucher disease, the most common sphingolipidosis, is caused by a deficiency of the lysosomal hydrolase glucocerebrosidase (D-glucosyl-N-acylsphingosine glucohydrolase; EC 3.2.1.45) (1–3). On the basis of clinical signs and symptoms, patients have been grouped into three distinct phenotypes. Type 1 (chronic nonneuronopathic) is found with increased frequency among the Ashkenazic Jewish population. In contrast there is no ethnic predilection of types 2 (acute neuronopathic) or 3 (chronic neuronopathic) Gaucher disease. The phenotypic-specific polymorphisms of glucocerebrosidase (4–9), the absence of functional complementation between the types in somatic cell hybridization studies (10, 11), and the ethnic predilection of only type 1 Gaucher disease (12) suggested that the three phenotypes of this disorder are a result of multiple allelic mutations.

We have reported (13) the isolation and characterization from a type 2 Gaucher patient of a genomic clone that contained a mutation (Leu-444 to Pro) that is frequently found in neuronopathic Gaucher disease. Only 20% of type 1 patients had the mutation, and then only in one allele.

Since type 1 Gaucher disease is the most prevalent type, investigators have hoped that identification of the mutations in the type 1 gene would provide a basis for diagnostic tests useful for genetic counseling. We now report the identifica-

tion of a mutation in an Ashkenazic Jewish patient with type 1 Gaucher disease that also occurs frequently in type 1 patients of other ethnic groups. The absence of this mutation in our patients with type 2 or 3 Gaucher disease or in normal controls, as well as the functional consequences of this mutation, are demonstrated.

MATERIALS AND METHODS

Study Population. The 12 unrelated normal control individuals of various ethnic origin ranged in age from 25 to 45 years. One control was of Ashkenazic origin. The 24 patients with non-neuronopathic Gaucher disease (type 1) ranged in age from 7 to 55 years and included 8 subjects of Ashkenazic origin. The 6 patients with type 2 disease ranged in age from 8 months to 2.5 years, and the 11 patients with type 3 disease ranged in age from 7 to 14 years. All patients, except two with type 2 disease, were seen at the National Institutes of Health under informed consent. The GM1260 and GM877 type 2 Gaucher fibroblast cell lines were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ).

DNA Preparation. High molecular weight DNA was prepared from autopsy or biopsy tissues, mononuclear blood cells, or cultured skin fibroblasts as described (13).

Clone Isolation and Nucleotide Sequence Analysis. A genomic library was constructed from an Ashkenazic Jewish patient with type 1 Gaucher disease. The procedures for the construction of the genomic library with DNA isolated from cultured skin fibroblasts as well as the cloning of the glucocerebrosidase gene have been described in detail elsewhere (13, 14). The *Bam*HI fragments of the glucocerebrosidase genomic DNA were subcloned into pUC19. Nucleotide sequence of all exons, splice junctions, and 5'- and 3'-flanking regions of the type 1 glucocerebrosidase gene were determined by the dideoxynucleotide chain-termination method (15–17).

Oligonucleotide-Directed *in Vitro* Mutagenesis and Transient Expression. The mutation (adenosine to guanosine transition) identified in exon 9 of the glucocerebrosidase gene isolated from the Ashkenazic Jewish type 1 Gaucher patient was introduced into a normal full-length cDNA clone, pcDGC8, isolated from an Okayama–Berg pcDX library constructed from simian virus 40-transformed normal human fibroblasts (18). A 20-mer oligonucleotide containing the adenosine to guanosine mutation was synthesized by the phosphoramidite method with an automated DNA synthesizer (model 380A, Applied Biosystems, Foster City, CA) (19, 20). The mutation sequence was introduced into the

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single-stranded DNA of the M13 subclone M13GCHB by the method of Zoller and Smith (21). A *HindIII*-*Mst* II fragment, M13GCHBT1, of the resultant construct was isolated and ligated to an *Mst* II- and partially *HindIII*-cleaved pcDGC8. The final construct pcDGCT1 codes for serine instead of asparagine at position 370 but otherwise is identical to pcDGC8.

COS cells were transfected with pcDGC8 or pcDGCT1 DNA or were mock-transfected by using the DEAE-dextran procedure (22). Forty-eight hours after transfection, cells were harvested, extracts were made, glucocerebrosidase activity was determined (4), and immunoblots were done by using anti-glucocerebrosidase rabbit polyclonal antibody (4).

Allelic-Specific Hybridization. The adenosine to guanosine mutation in the glucocerebrosidase gene in type 1 patients was identified with oligonucleotide probes, because no restriction endonuclease sites were affected. A 19-mer oligonucleotide with the normal sequence (5' TACCCTA-GAACCTCCTGT 3', probe A), a 19-mer oligonucleotide with the adenosine to guanosine substitution (5' TACCCTA-GAGCCTCCTGT 3', probe B), and an 8-mer primer oligonucleotide (5' ACAGGAGG 3') were synthesized as described above. High specific-radioactivity probes used in the allelic-specific hybridization experiments were synthesized by primer extension reactions (23, 24).

Southern blots of *Bam*HI digests of total human genomic DNA were hybridized with probe A (normal probe) at 53°C or probe B (mutant probe) at 51°C in 6× SSC containing 10× Denhardt's solution, 0.05% sodium pyrophosphate, and tRNA (2 μg/ml) for 18 hr (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0; 1× Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin). The filters were washed at room temperature for 60 min, at 37°C for 30 min, and finally at 55°C for 3 min for probe

A or at 53°C for probe B in 6× SSC/0.05% sodium pyrophosphate.

Protein Secondary Structure Prediction. Secondary structure was predicted by using commercially available software (MicroGenie, Beckman).

RESULTS

Identification of a Gene Mutation in an Ashkenazic Jewish Type 1 Patient. A genomic clone, T1BB, was isolated from a library constructed from DNA from cultured skin fibroblasts of an Ashkenazic Jewish type 1 patient. Both parents of the patient were also Ashkenazic. The genomic clone had a 14-kilobase (kb) insert and showed an identical restriction map to the normal genomic clone[§] (PCN9) (25). Nucleotide sequence analysis of all exons, splice junctions, and 5'- and 3'-flanking regions revealed only a single-base change (adenosine to guanosine) in exon 9 of the type 1 glucocerebrosidase gene, resulting in the substitution of serine for asparagine at position 370 (Fig. 1). Computer modeling predicted that from amino acid 366 to amino acid 377 the normal protein would have a β-pleated sheet structure with an interruption at asparagine-370. In contrast, the entire region from amino acid 366 to amino acid 377 in the mutant protein containing serine at position 370 is predicted to be a β-pleated sheet.

Oligonucleotide-Directed Mutagenesis and Transient Expression. A full-length mutant cDNA clone, pcDGCT1 (coding for serine at amino acid position 370), was constructed as described above. As shown in Table 1, COS cells transfected with the mutant cDNA pcDGCT1 had no significant increase

[§]The cDNA sequence of exon 9 reported in this paper has been deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. K02920).

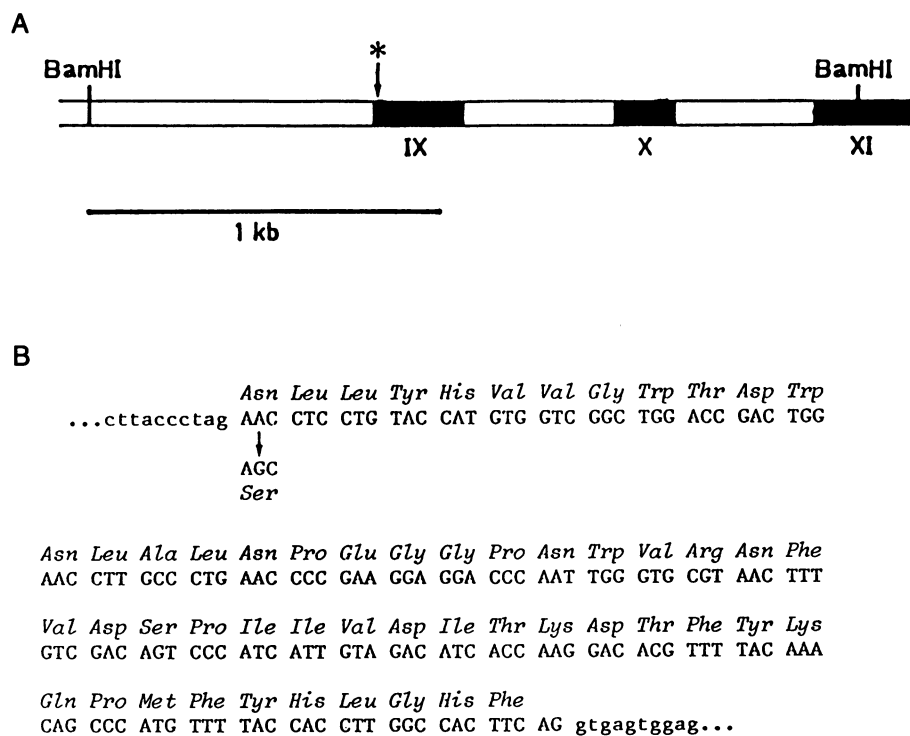


FIG. 1. Schematic diagram of exons 9–11, including the flanking introns (A), and the nucleotide sequence of exon 9 (uppercase roman letters) and its flanking intervening sequences (lowercase roman letters), and the amino acid sequence (italic letters) (B) are shown. (A) Exons and introns are indicated by solid and open areas, respectively. The location of the single-base substitution (adenosine to guanosine) in exon 9 of the type 1 genomic DNA is indicated by the asterisk. (B) Single-base substitution (adenosine to guanosine) that was found after a comparison of the nucleotide sequences of the entire coding sequence, splice junctions, and the 5'- and 3'-flanking regions of the normal and type 1 genes is shown. This base change results in the substitution of serine for asparagine at amino acid position 370.

Table 1. Expression of glucocerebrosidase activity in transfected COS cells

	Glucocerebrosidase specific activity, units*/mg	% increase
Mock	291	—
pcDGC8	742	155
pcDGCT1	297	2

*Units are expressed as nmol produced per hr at 37°C (4).

in glucocerebrosidase activity. In contrast, COS cells transfected with the normal cDNA clone pcDGC8 had a 155% increase in glucocerebrosidase activity. Immunoblot analysis of COS cells transfected with the mutant cDNA showed a pattern of cross-reacting material identical to COS cells transfected with the normal cDNA pcDGC8.

Allele-Specific Hybridization. Analysis of an Ashkenazic Jewish family was performed with probes synthesized by primer extension (Fig. 2). The 2.2-kb *Bam*HI fragment originated from the functional glucocerebrosidase gene, and the 1.8-kb fragment originated from the pseudogene (14).

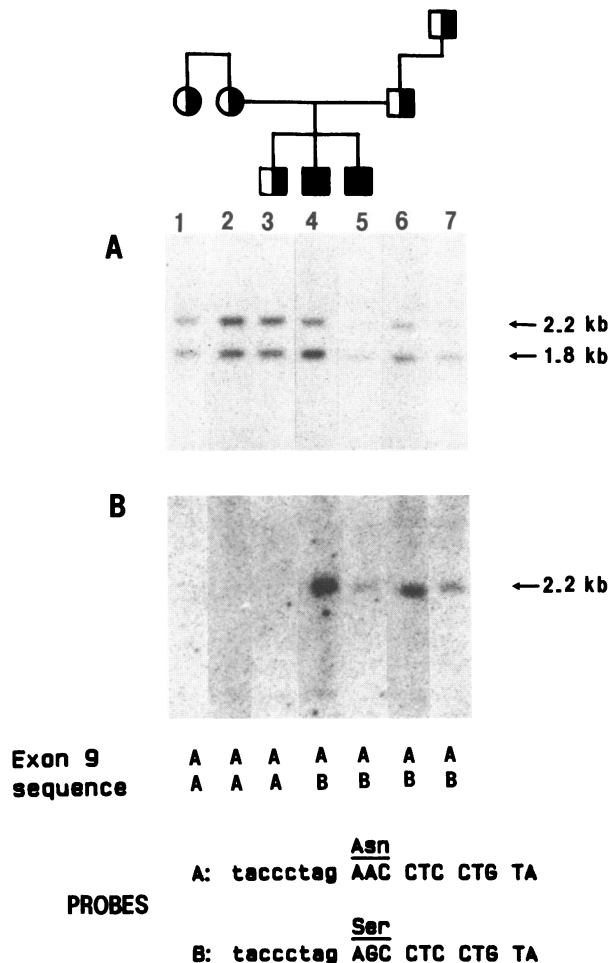


FIG. 2. Allele-specific hybridization analysis of genomic DNA from the Ashkenazic patient with type 1 Gaucher disease (lane 4) and his family members (lanes 1–3 and 5–7). The pedigree chart of the family is included above the Southern blots (carriers, half-solid symbols; homozygotes, solid symbols). *Bam*HI-digested genomic DNA was hybridized to either the normal (A) or to the mutant (B) oligonucleotide probe prepared by primer extension. The genotypes determined by the sequence in exon 9 and the sequences of the two probes are shown under the autoradiogram; the letters A and B denote the presence of asparagine and serine, respectively, at amino acid 370. The 2.2-kb and 1.8-kb *Bam*HI fragments originate from the functional gene and pseudogene, respectively.

The 2.2-kb *Bam*HI from the patient's DNA hybridized to both probes A and B, indicating that the patient has one allele with Ser-370 and another allele with Asn-370. Since only the father and paternal grandfather are identified as having the mutant allele with Ser-370, it is clear that this mutation has been transmitted from the paternal grandfather through three generations. Furthermore, the patient and his mother must both have an allele with another as yet unidentified mutation in the glucocerebrosidase gene.

By using oligonucleotide probes A and B we screened 24 type 1 patients, 6 type 2 patients, and 11 type 3 patients, and 12 normal controls. The results are summarized in Table 2. None of the normal controls, the studied type 2 patients, or type 3 patients had this mutant allele. In contrast, 15 type 1 patients had one allele with the mutation, and 3 additional patients were homozygous for the mutation. Interestingly, 1 of the type 1 patients had one allele with the Asn-370 to Ser mutation and another allele with the Leu-444 to Pro mutation that had been described (13) in patients with neuronopathic Gaucher disease.

DISCUSSION

Investigators have suggested that the Ashkenazic Jews with type 1 Gaucher disease might have a single-gene mutation (26). However, studies have provided evidence for genetic heterogeneity in the Ashkenazic Jewish population with type 1 Gaucher disease (27). Linkage analysis by using restriction fragment length polymorphisms has also suggested the possibility that different mutations in the glucocerebrosidase gene result in type 1 Gaucher disease in the Ashkenazic Jews (28).

Our present study demonstrates that there are at least four genotypes of the glucocerebrosidase gene in type 1 Gaucher disease. These genotypes are (i) Ser-370/Ser-370, (ii) Ser-370/Pro-444, (iii) Ser-370/X, and (iv) X/X, where X is a mutation that has not yet been identified. In the Ashkenazic Jewish population we found three genotypes (i–iii). This clearly indicates that there is more than one allelic mutation in the glucocerebrosidase gene in Ashkenazic Jews. Furthermore, the Asn-370 to Ser mutation is frequently found in both Ashkenazic and non-Jewish type 1 Gaucher patients. The increased frequency of type 1 Gaucher disease in Ashkenazic Jews is likely a consequence of a "founder effect" rather than a selective advantage of the heterozygote, because although both Jewish and non-Jewish patients have the Asn-370 to Ser mutation, the type 1 phenotype is more common only in the Ashkenazic population.

For the reported Leu-444 to Pro mutation we suggested homologous recombination between the functional gene and a pseudogene as a possible mechanism of the gene mutation (13), because the pseudogene contained the same mutation sequence. Allele-specific hybridization and nucleotide sequence analysis of the pseudogene demonstrated that the pseudogene has a nucleotide sequence coding for asparagine rather than serine (unpublished data). Although deamination of the methylcytosine of a CpG dimer to thymidine is a

Table 2. Frequency of Asn-370 to Ser mutation in 41 patients with Gaucher disease and 12 normal controls

Subjects	Genotype, no.		
	+/+	+/-	-/-
Normal controls	12	0	0
Patients with Gaucher disease			
Type 1 (nonneuronopathic)	6	15	3
Type 2 (acute neuronopathic)	6	0	0
Type 3 (chronic neuronopathic)	11	0	0

A plus sign denotes the presence of the normal sequence, and a minus sign denotes the presence of the Asn-370 to Ser mutation.

mutation hot spot of cytidine to thymidine transition (29), we cannot explain the Asn-370 to Ser mutation by either this mechanism or homologous recombination.

Although it is possible that the adenosine to guanosine mutation in exon 9 near the junction of intron 8 could have resulted in an abnormal splicing with an attendant reading frame shift, a comparison of glucocerebrosidase biosynthesis in normal and type 1 Gaucher fibroblasts by immunoblot (4) and by pulse-chase analyses (30) suggests that this has not occurred. The same biosynthetic forms are seen in fibroblast extracts from type 1 Gaucher patients and normal controls. However, the 59-kDa protein biosynthetic form from type 1 fibroblasts was less stable than that from control fibroblasts (30).

We have reported that the Leu-444 to Pro mutation occurred frequently in type 2 and type 3 Gaucher disease and that all patients homozygous for the Leu-444 to Pro mutation had neurologic abnormalities (13). The Asn-370 to Ser mutation described in this report is found exclusively in type 1 Gaucher patients. This suggests that the identification of these mutations will be useful for diagnostic testing and genetic counseling. Furthermore, the fact that one type 1 adult patient had both the Asn-370 to Ser and Leu-444 to Pro mutations suggests that the presence of mutant glucocerebrosidase coded by the allele having the Asn-370 to Ser mutation might prevent patients from developing central nervous system abnormalities. These findings also suggest that the glucocerebrosidase mRNA and protein within a cell may be heterogeneous and represent that encoded by each allele. Thus, biochemical and kinetic characterizations of glucocerebrosidase in a patient's cells must be interpreted with this in mind. Also, the pathogenetic mechanisms of central nervous system involvement in patients homozygous for the Leu-444 to Pro mutation are still not clear.

Thus far, two mutations in the glucocerebrosidase gene have been identified in Gaucher patients, one common in type 1 and the other in types 2 and 3. The information obtained from allele-specific hybridization and restriction fragment length polymorphism analysis of these two mutations should be useful for diagnosis and genetic counseling, since $\approx 80\%$ of our Gaucher patients appear informative with respect to these two mutations. Further definition of other mutations causing Gaucher disease should lead to a more complete understanding of the genetics and biochemistry of this disorder.

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