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Mutation Analysis of Gaucher Disease Patients From Argentina: High Prevalence of the Rec*Nci*I Mutation

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Gaucher disease (GD) is caused by a deficiency of β-glucocerebrosidase activity mainly due to mutations in the gene coding for the enzyme. More than 100 mutations have been identified to date and their frequencies have been established in several populations, including Ashkenazi Jews, among whom the disease is particularly prevalent. In order to study the molecular pathology of the disease in patients from Argentina, we conducted a systematic search for mutations in the glucocerebrosidase gene. Genomic DNA from 31 unrelated GD patients was screened for seven previously described mutations: N370S (1226A→G), L444P (1448T→C), D409H (1342G→C), R463C (1504C→T), 1263del55, Rec*Nci*I, and RecTL. This allowed the identification of 77.4% of the GD alleles: N370S and Rec*Nci*I were the most prevalent mutations found (46.8% and 21% respectively). Southern analysis demonstrated three distinct patterns for the Rec*Nci*I alleles. In order to identify the remaining alleles, the full coding region of the gene, all the splice sites, and part of the promoter region were analyzed by single-strand conformational polymorphism analysis (SSCP) after polymerase chain reaction amplification. This extensive screening allowed the identification of 13 different mutations, accounting for 93% of the total number of GD alleles. Three novel missense mutations, I161S (599T→G), G265D (911G→A), and F411I (1348T→A), were detected. Twelve polymorphic sites within the glucocerebrosidase gene are in complete linkage disequilibrium and define two major haplotypes, "–" and "+". Mutation N370S was always associated with the "–" haplotype, as described in other populations. Interestingly, the Rec*Nci*I alleles with the same Southern-blot pattern were always associated with the same haplotype. Am. J. Med. Genet. 80:343–351, 1998.

KEY WORDS: Gaucher disease; glucocerebrosidase gene; mutation screening; Argentinian patients; Rec*Nci*I mutation

INTRODUCTION

Gaucher disease (GD) is the most common lysosomal storage disorder. It is inherited as an autosomal recessive trait, caused by a deficiency of the enzyme β-glucocerebrosidase (GBA), which leads to the accumulation of glucocerebroside in various tissues [Beutler and Grabowski, 1995]. All cases described to date are due to mutations in the glucocerebrosidase gene, except in two patients who had a defect of the saposin gene, which encodes an activating protein (SAP-C) for β-glucocerebrosidase [Schnabel et al., 1991; Rafi et al., 1993]. Three clinical types have been defined according to the absence (Type I, MIM 230800) or presence and severity of neurological involvement (Types II and III, MIM 230900 and 231000, respectively).

The disease is most common in the Ashkenazi Jewish population (frequency about 1/850) [Beutler and Grabowski, 1995], while the prevalence in other populations is lower (1/40,000–60,000) [Grabowski, 1993].

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Among Ashkenazi Jews mutations N370S (1226A→G) and 84GG account for approximately 77% of the mutant alleles according to data from most groups [reviewed in Beutler and Grabowski, 1995]. Although to a lesser extent, mutation N370S is also the most frequent in most of the populations analyzed, while mutation L444P (1448T→C) is the second most common [Walley et al., 1993; Lewis et al., 1994; Cormand et al., 1995; Ida et al., 1995; Amaral et al., 1996; le Coutre et al., 1997]. Besides single nucleotide changes in the glucocerebrosidase gene, complex mutant alleles, which include several point mutations, have been described. These point mutations are present in a highly homologous pseudogene [Horowitz et al., 1989], which is located 16 kb down-stream from the glucocerebrosidase gene at chromosome band 1q21. Unequal crossing over between the gene and the pseudogene or gene conversion events have been suggested as possible mechanisms by which these complex alleles are generated [Zimran and Horowitz, 1994]. The most prevalent complex alleles are Rec*Nci*I, including aminoacid changes L444P and A456P (1483G→C), and the silent nucleotide change at codon 460 (V460V, 1497G→C), and RecTL, with the same changes as Rec*Nci*I plus mutation D409H (1342G→C).

In addition to the disease-causing mutations, 12 polymorphic sites have been described in the introns and flanking regions of the glucocerebrosidase gene, which are in linkage disequilibrium and give rise to only two major haplotypes named "+" and "–" [Beutler et al., 1992b].

Here we present an extensive mutation analysis of 31 GD patients of varying ancestry diagnosed in Argentina. More than 93% of the mutant alleles were identified and a high prevalence of the complex allele Rec*Nci*I was found. To our knowledge, this is the first report on Argentinian GD patients, with the exception of three patients described by Argaraña et al. [1995].

MATERIALS AND METHODS

Patients

Mutation analysis was performed on 31 unrelated GD patients and a number of their relatives: 29 were from Argentina, one was from Chile, and one from Paraguay. Patients A5, A21, A46, A48, and A60 have Ashkenazi Jewish ancestries. The origin of the patients was defined by the place of birth of the grandparents. Patients whose grandparents were born in Argentina were considered to have an Argentinian origin. However, it should be noted that the Argentinian population is very heterogeneous, and most of its people are of European ancestries. Most of the GD patients defined as "Argentinian" bear a Spanish surname indicating that at least one of the ancestors was a Spaniard.

The diagnosis of GD was established by demonstration of low β-glucocerebrosidase activity in leukocytes. In addition, a clinical evaluation was provided by the patient's physician. Most of the patients (30/31) were classified as Type I, although later neurological involvement cannot be ruled out in some of them due to their young age. Information on ethnic background was provided by the patients.

DNA Isolation

Genomic DNA was prepared from peripheral blood leukocytes using the salting out procedure [Miller et al., 1988].

Detection of Seven Previously Described Glucocerebrosidase Gene Mutations

All patients were screened for four known missense mutations in the glucocerebrosidase gene (N370S, L444P, D409H, R463C), for the complex alleles RecTL and Rec*Nci*I, and for the 55-bp deletion 1263del55. Mutations N370S, L444P, and R436C were analysed by polymerase chain reaction (PCR) amplification and restriction enzyme digestion as previously described [Cormand et al., 1995]. Mutations D409H and A456P, present in the recombinant alleles, were detected by allele-specific oligonucleotide (ASO) hybridization as described in Cormand et al. [1995], using the following primers and hybridization temperatures: D409H: 5′-ATCACCAAGGACACGTTTT-3′ (normal), 59°C; 5′-ATCACCAAGCACACGTTTT-3′ (mutant), 55°C; A456P: 5′-GATGGCTCTGCTGTTGTGG-3′ (nor), 60°C; 5′-GATGGCTCTCCTGTTGTGG-3′ (mut), 60°C (the allele specific nucleotide is underlined). Mutation D409H was additionally studied by single-strand conformation polymorphism (SSCP) analysis [Chabás et al., 1996]. The detection of the silent change V460V, present in both recombinant alleles, was carried out according to Latham et al. [1990].

The 1263del55 mutation was screened by gene-specific PCR amplification of a 476-bp fragment (forward primer, nt 5569–5588; reverse primer, nt 6025–6044) and separation of the products on a 2% agarose gel.

PCR Amplification and SSCP Analysis

PCR amplification and SSCP analysis of 14 DNA fragments covering the eleven exons of the glucocerebrosidase gene were performed from genomic DNA of the patients. The size of the PCR fragments ranged from 139 bp to 292 bp (Table I), in order to achieve optimal results in the SSCP analysis [Orita et al., 1989]. All primer pairs were chosen to amplify the gene and not the highly homologous pseudogene, except in exon 11, for which this was not possible. For gene-specific amplification of the latter exon, a nested PCR was performed from a larger, gene-specific PCR fragment (primers 10-F and 11-R).

Although many primers presented several mismatches with the pseudogene sequence, in six of the primer pairs, one of the primers was nonspecific while the other presented only one mismatch in the last nucleotide (3′), in relation to the pseudogene sequence. Before the systematic mutation scanning, each of the 14 fragments was amplified on genomic DNA from a normal individual, cloned, and sequenced. The sequences of 15 clones from each product showed that, in all cases, only the gene and not the pseudogene was amplified.

For all fragments, the PCR reaction was performed under the following conditions: 100 ng of template DNA, 1 U of Dynazyme DNA polymerase (Finnzymes

TABLE I. Primers for PCR Amplification of the Glucocerebrosidase Gene and Product Sizes

Exon[a]	Primer sequence (5′→3′)[b]	Genomic position[c]	Size (bp)
1a	F. ATCCTCTGGGATTTAGGAGC*	222–241	264
	R. CTTAGCTATAGGCACTAGGT	466–485	
1b	F. GCCGGAATTACTTGCAGGGC*	444–463	202
	R. CTGTGACAATGCTGATTGGG	626–645	
2	F. AGGCAGCTAAGCCCTGCCCA*	898–917	215
	R. AGAAGGGAGGCTCTGTGCTA	1093–1112	
3	F. CAGACCTCACTCTGCTTGTA*	1585–1604	271
	R. GGAGGACCCAGCCTGGCCCA*	1836–1855	
4	F. TGGGTACTGATACCCTTATT*	1897–2016	223
	R. TCAATGGCTCTATGTCATCT*	2100–2119	
5	F. ACCCAGGAGCCCAAGTTCCC*	2916–2935	288
	R. CCTCAGGGCCTGAAAAAGCT	3184–3203	
6	F. CTCTGGGTGCTTCTCTCTTC	3346–3365	271
	R. ACAGATCAGCATGGCTAAAT*	3597–3616	
7a	F. CTCGGCTTCCCAAAGTGCTG*	4010–4029	250
	R. CTAGGTCACGGGCAATGAAG	4240–4259	
7b	F. TGGGCTTCACCCCTGAACAT	4212–4231	232
	R. ATAGTTGGGTAGAGAAATCG*	4424–4443	
8	F. TGTGCAAGGTCCAGGATCAG*	5179–5198	292
	R. TTTGCAGGAAGGGAGACTGG	5451–5470	
9a	F. GTGTTGAGCCTTTGTCTCTT	5800–5819	139
	R. GATGGGACTGTCGACAAAGT*	5919–5938	
9b	F. ACTGGAACCTTGCCCTGAAC*	5871–5890	174
	R. ATAGGCCTGGTATGGAATGG	6025–6044	
10	F. GAGAGCCAGGGCAGAGCCTC*	6253–6272	291
	R. AGGCCCCCAACGCTGTCTTC	6524–6543	
11	F. GGATCACACTCTCAGCTTCT	6549–6568	227
	R. CTCTTTAGTCACAGACAGCG	6756–6775	

[a]"a" and "b" indicate overlapping 5′ and 3′ regions of an exon, respectively.
[b]Asterisks indicate gene-specific primers; F, forward primer; R, reverse primer.
[c]According to Horowitz et al. [1989].

Oy), 200 μM dNTPs, and 20 pmol of each primer in the recommended buffer in a final volume of 50 μl. The PCR program consisted of 35 cycles of denaturation at 94°C for 30 sec and a unique annealing/extension step at 55°C for 30 sec.

The SSCP analysis was performed as described in Cormand et al. [1997a]. Four SSCP conditions were tested for each fragment, combining different polyacrylamide concentrations (8 or 12% acrylamide:bisacrylamide 29:1), different glycerol concentrations in the gel (0% or 5% glycerol), and two running conditions (RT at 200 V, or 4°C at 300 V, always 16 hr).

Cloning and Sequencing

The PCR products showing abnormal SSCP patterns were cloned and sequenced as previously described [Cormand et al., 1996]. In each case, six to ten clones were sequenced by fluorescence dideoxy cycle sequencing (ABI 373A Fluorescent DNA sequencer, Perkin Elmer). Authenticity of putative mutations was confirmed either by restriction analysis or by direct sequence of genomic DNA PCR products.

Southern Blot, Probe Preparation, and Hybridization

Genomic DNA from the patients was digested with *Ssp*I, electrophoresed on a 0.7% agarose gel, and blotted onto a nylon membrane (Amersham) using standard protocols.

For probe preparation, total RNA was prepared from human cultured fibroblasts by the Ultraspec RNA Isolation System (Biotecx). Reverse transcription was performed using the Time Saver cDNA Synthesis Kit (Pharmacia Biotech) with an antisense primer at the 3′ end of the glucocerebrosidase mRNA (5′-CTCTTTAGTCACAGACAGCG-3′). The full coding region of the cDNA was PCR-amplified in a single 1836-bp fragment using the reverse transcription primer and a sense primer at the 5′ end of the cDNA (5′-GCCGGAATTACTTGCAGGGC-3′). The PCR reaction was performed with the Expand Long Template PCR System (Boehringer Manheim).

The glucocerebrosidase cDNA probe was labelled with α^{32}P-dCTP using the random priming procedure. Southern blot hybridization was carried out at high stringency following a standard protocol. The membrane was exposed to a Hyperfilm-MP (Amersham) for 3 days.

Analysis of the 6144A→G Polymorphism

The 6144A→G polymorphism in intron 9 of the glucocerebrosidase gene was analysed by *Hha*I digestion of a PCR-amplified product. Gene-specific primers (forward: nt 5904–5923, reverse: 6655–6690) were used to amplify a 787-bp fragment. The alleles were designated as "+" (*Hha*I site present, "G") or "–" (*Hha*I site absent, "A"), according to Beutler et al. [1992b]. It should be noted that these authors used enzyme *Bgl*I. Both *Hha*I and *Bgl*I sites are destroyed if an "A" is present in the sequence.

Association of the mutations with either the "+" or the "–" variants of the polymorphism was studied. In

double heterozygous patients, phase was established by genotyping the parents if available.

Statistical Analyses

Allelic association between the *Hha*I polymorphism and the common N370S and L444P mutations was evaluated using the $D' = D/D_{max}$ standardized linkage disequilibrium coefficient [Lewontin 1988]. The significance of the association was tested by a one-sided χ^2 test.

The homozygosity (j) at the *GBA* locus in a given population was determined by $j = \sum x_i^2$, where x_i is the frequency of the ith GD allele [Guldberg et al., 1996]. Since in most populations studied the identification of GD mutations was not 100%, we have calculated maximum and minimum values for the homozygosity index, either considering all the unidentified alleles as a single one or assuming that all the unknown alleles are different, each having a frequency of 1/N, where N is the total number of mutant chromosomes studied.

RESULTS

Mutation Analysis

Mutation analysis was carried out on 31 unrelated patents with GD, mainly from Argentina. An initial screening for seven previously described mutations in the glucocerebrosidase gene—N370S (1226A→G), L444P (1448T→C), D409H (1342G→C), R463C (1504C→T), 1263del55, Rev*Nci*I, and RecTL–allowed the identification of 77.4% of the mutant alleles. Mutations R463C, 1263del55, and RecTL were not detected among these patients. The most frequent mutation was the missense mutation N370S (29/62 GD alleles) identified in two homozygous patients and 25 compound heterozygotes.

The gene-pseudogene recombinant allele Rec*Nci*I was the second most frequent GD mutation in our series of patients (13/62 mutant alleles). In order to investigate the molecular mechanism of these gene-pseudogene recombinant alleles we performed Southern blot analysis of genomic DNA digested with the restriction endonuclease *Ssp*I (Fig. 1). This analysis was performed on 12 out of the 13 Rec*Nci*I unrelated chromosomes (and in one sib, A44). Our results provide evidence for three distinct rearrangements. The *Ssp*I digestion reveals two bands of ~18 and 12 kb in normal chromosomes, corresponding to the gene and the pseudogene respectively [Zimran et al., 1990b]. This pattern was observed in five cases (Patients A17, A22, A50, A57, A59), indicating that either gene conversion or a double recombination event has occurred. In one case (Patient A48), an additional band of 14 kb was obtained, suggesting that a fusion gene has been created through an unequal crossing over between the functional glucocerebrosidase gene and its highly homologous pseudogene [Zimran et al., 1990b]. A third pattern composed of three bands of ~18, 16, and 12 kb was observed in six unrelated individuals.

In order to identify the remaining GD alleles, we performed SSCP analysis on PCR products covering exons 1 to 11 of the glucocerebrosidase gene, the flanking intron-exon junctions and part of the promoter sequence. This extensive analysis showed that 13 different mutations account for 93% of the mutated alleles. Segregation of most mutations in the families was studied and normal Mendelian inheritance was observed in all cases.

Table II shows the genotypes found for all the patients. Age of onset and current age, some clinical findings and the geographic or ethnic origin are also included. Patient A1 presents the mutations that are found in the recombinant allele named RecTL. However, the analysis of the DNA from his parents showed that he bears the D409H mutation in the chromosome inherited from his mother and the Rec*Nci*I mutations in the chromosome inherited from his father. The relative frequencies of the most common mutations identified are shown in Figure 2.

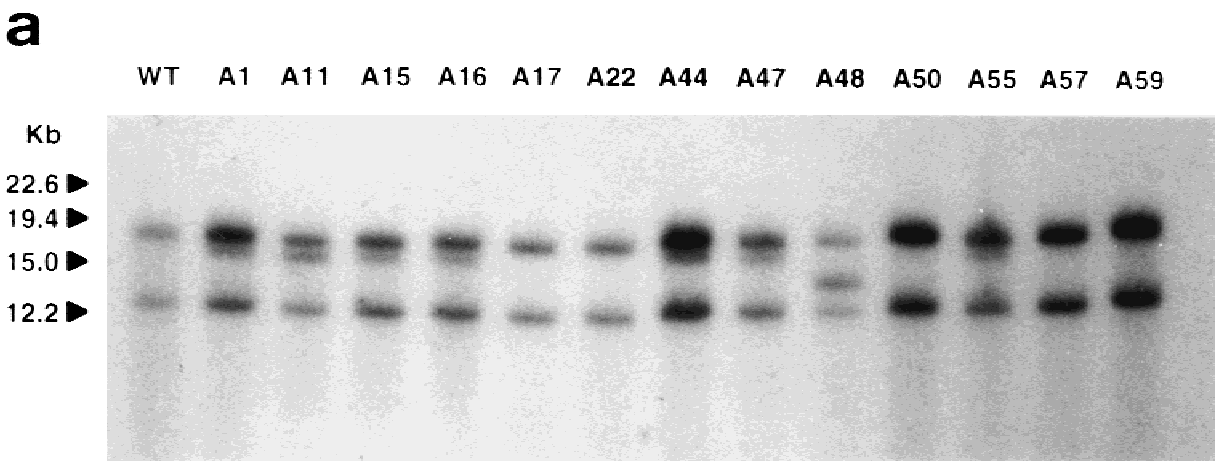
Eight of the 13 mutations were found only once (Table III), three of which, I161S (599T→G), G265D (911G→A), and F411I (1348T→A), had not been described before. The detection of one of these new mutations, G265D, by SSCP is shown in Figure 3. These mutations were not found in a screening of 80 normal chromosomes. The missense mutation I161S changes a nonpolar amino acid (isoleucine) to a polar one (serine), due to a T-to-G transversion at genomic nucleotide 3393 in exon 6. Mutation G265D changes an uncharged polar amino acid (glycine) to an acidic amino acid (aspartic acid), due to a G-to-A transition at genomic nucleotide 4260 in exon 7. The I161S and G265D mutations are present in the heterozygous state with the common N370S allele in patients A27 and A31, respectively. The segregation in the family could not be studied for patient A27 as no samples were available from the parents. The third novel mutation identified was a T-to-A transversion at genomic nucleotide 5963 in exon 9. This mutation (F411I) predicts a substitution of a isoleucine in the mutant for a phenylalanine in the normal allele. In this case, the affected member (A51) was heterozygous for mutation D409H.

Allelic Heterogeneity

Allele frequencies were used to compare the mutation heterogeneity at the *GBA* locus among different populations with at least 70% of the mutant alleles identified (Table IV), using the homozygosity index as described in Guldberg et al. [1996]. Values ranged from a maximum of 1 for the Norrbottnian GD patients who all have the same mutation (L444P) at homozygosity, to 0.17 in Australasia, and 0.16 in a mixed non-Jewish population. The Argentinian GD population has an intermediate value of 0.29.

Allelic Associations

The analysis of the 6144A→G polymorphism in intron 9 of the glucocerebrosidase gene showed that 21 out of 29 N370S alleles are unequivocally associated with the "–" variant of the polymorphism in our group of patients. The remaining eight N370S alleles were present in heterozygosity with another mutation in patients with the +/– genotype. Although phase was not established in these cases, these results are consistent with the association. In contrast, among four L444P



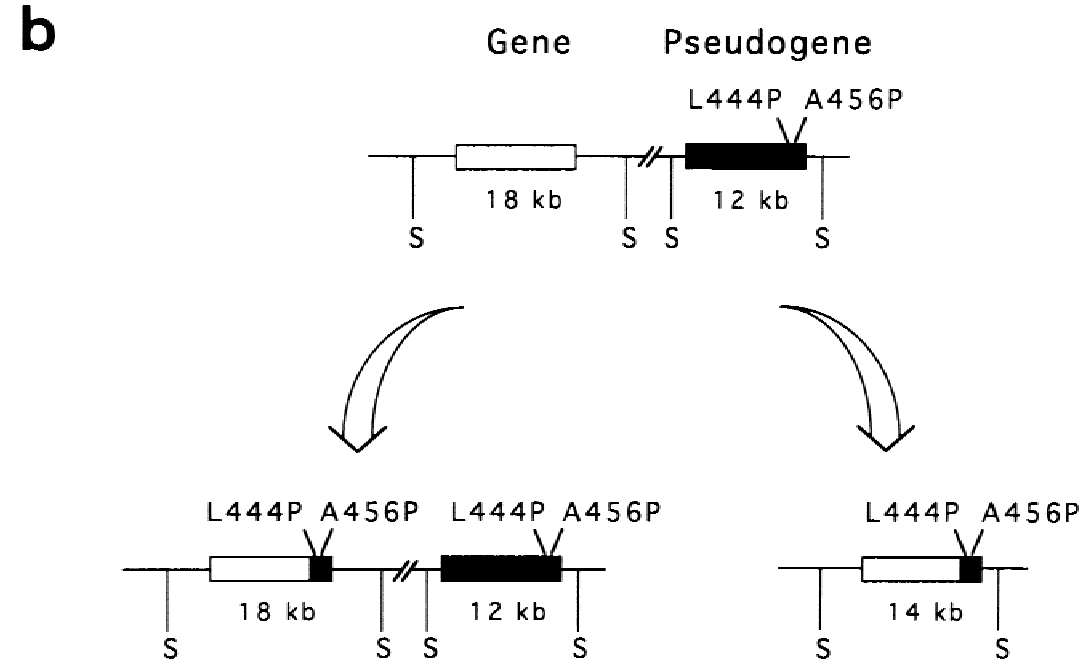


Fig. 1. **a:** Southern blot analysis of genomic DNA samples digested with *Ssp*I. The lane on the left is a normal control with two bands of ~18 and 12 kb. Patients A17, A22, A50, A57, and A59 have the normal two-banded pattern. Patients A1, A11, A15, A16, A44 (and his brother A47) and A55 show an extra 16-kb band. Patient A48 has the 18- and 12-kb bands plus an additional 14-kb band. **b:** Putative models for the different Rec*Nci*I alleles. Those showing a ~18–12-kb pattern (*left*) could have arisen from gene conversion while the allele showing the 14-kb band (*right*) could be the product of an unequal crossing over giving rise to a fusion gene. This allele is often mentioned as XOVR.

alleles found, two were associated with the "+" allele and two with the "–" allele.

In addition, 44 nonaffected individuals, 10 from Argentina and the rest from Spain, were genotyped in order to determine the frequency of the two haplotypes in a normal population. The "–" haplotype accounts for 70.5% (62/88) of the chromosomes studied, and the "+" haplotype for 29.5% (26/88), in agreement with previous reports [Sorge et al., 1985; Glenn et al., 1994]. These data were used to evaluate the degree of linkage disequilibrium between the N370S mutation and the common "–" haplotype, and highly significant values were obtained ($D = 0.046$, $D' = 1$, $P < 0.005$).

Interesting allelic associations were found in the Rec*Nci*I chromosomes. All of the recombinant alleles showing the two-banded pattern in the Southern blot analysis were found associated with the uncommon "+" variant. A positive linkage disequilibrium was found

TABLE II. Genotypes, Clinical Findings and Origin of the Patients

Patient	Genotype	*Hha*I polymorphism[a]	Age[b]	First symptoms[c]	Type	Geographic/ethnic origin[d]
A1	D409H/Rec*Nci*I	–/–	10m (†)	S	II	Paraguayan/Spanish
A4	N370S/L444P	–/–	36y (41y)	S, epitaxis	I	Spanish/Italian
A5,A6[e]	N370S/84GG	–/+[f]	2, 3y (18y,18y)	S, SH	I	2 Spanish/Polish (AJ)/Russian (AJ)
A9	N370S/R120W	–/+[f]	1y	S	I	Spanish
A10	N370S/R285C	–/–	?	?	I	Mapuche
A11,12,61[g]	N370S/Rec*Nci*I	–/–	15y (19,18,17y)	Bone disease	I	Argentinian
A14	N370S/?	–/–	? (47y)	S	I	Italian
A15	N370S/Rec*Nci*I	–/–	1y (9y)	S	I	Argentinian/Paraguayan
A16	N370S/Rec*Nci*I	–/–	1y (3y)	SH	I	Argentinian
A17	N370S/Rec*Nci*I	–/+[f]	? (54y)	?	I	?
A18,19[g]	N370S/L444P	–/+[f]	6, 5y (15,10y)	Bone disease, S	I	Argentinian
A21	N370S/N370S	–/–	21y (66y)	S	I	Polish (AJ)/German (AJ)
A22	Rec*Nci*I/?	+/+	9m (10m)	S	I	Argentinian/Polish
A25	N370S/D399N	–/+	6y (12y)	S	I	?
A27	N370S/I161S	–/–	7y (8y)	S, adenopathy[h]	I	2 Spanish/Italian/Armenian
A28	N370S/Rec*Nci*I	–/–	46y	S	I	Argentinian
A31,32[g]	N370S/G265D	–/+	? (19y,12y)	S	I	Chilean
A40	N370S/?	–/+[f]	10y (14y)	SH	I	Argentinian
A42	N370S/L444P	–/+	2y (10y)	S	I	Spanish/Italian
A45	N370S/L444P	–/–	3y (4y)	S	I	Spanish/Arabic
A46	N370S/?	–/+[f]	4y (51y)	?	I	German (AJ)
A47, A44[g]	N370S/Rec*Nci*I	–/–	3y (4y), 8m	S	I	Argentinian
A48	N370S/Rec*Nci*I	–/+	1y (20y)	S	I	Ashkenazi Jewish
A50	RecNciI/R48W	+/–	3y (5y)	Bone disease	I	Argentinian
A51	D409/F411I	–/–	1y (2y)	SH	I	Argentinian
A54	N370S/N370S	–/–	30y (31y)	Icterius	I	Argentinian/Spanish
A55	N370S/Rec*Nci*I	–/–	6y (33y)	SH	I	Argentinian
A56	N370S/G202R	–/–	6y (20y)	S	I	2 Spanish/German/Italian
A57	N370S/Rec*Nci*I	–/+[f]	8m (4y)	SH	I	Spanish/Argentinian
A59	N370S/Rec*Nci*I	–/+	4y (6y)	S	I	Argentinian/Spanish
A60	N370S/84GG	–/+[f]	10m (47y)	S	I	Russian(AJ)

[a]6144A→G polymorphism in intron 9 of the *GBA* gene (A allele: "–"; G allele: "+").
[b]Age at presentation (present age or age of death†). y, years; m, months.
[c]S, Splenomegaly; H, Hepatomegaly.
[d]Geographic and ethnic origin of the grandparents; AJ, Ashkenazi Jewish. When three origins are mentioned it is indicated which one corresponds to two of the grandparents. Argentinian, Chilean, or Paraguayan mean that grandparents were born in these countries and no additional information is available.
[e]Dizygotic twins.
[f]Phase between the mutations and the intragenic *Hha*I polymorphism is not established (in all the other cases, mutations and *Hha*I alleles on the same side are present on the same chromosome).
[g]Sibs from the same family.
[h]Proband, unaffected sister, and mother presented with β-thalassemia.

between the two-band-pattern mutant allele and the "+" haplotype ($D = 0.036$, $D' = 1$, $P < 0.005$). In two cases (Patients A17 and A57) phase was established assuming that the accompanying mutation, N370S, was in a "–" chromosome. In contrast, when the 18–16–12 kb pattern was present, the Rec*Nci*I allele was associated with the "–" allele. No significant linkage disequilibrium was observed due to the association with the more common haplotype ("–"). The fusion gene found in individual A48 was associated with the "+" variant.

DISCUSSION

Mutation Analysis

Here we describe an extensive search for GD-causing mutations performed in 31 patients, mainly from Argentina, but with different geographic or ethnic origins, including some patients with Ashkenazi Jewish ancestries. This study allowed the identification of more than 93% of the mutant alleles. Mutation N370S alone accounts for almost half of the total number of

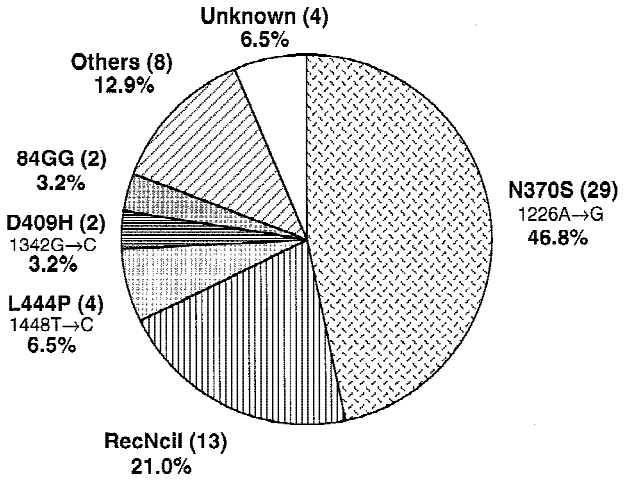


Fig. 2. Prevalence of mutations among 31 unrelated GD patients mainly from Argentina. The numbers in brackets represent number of GD alleles.

TABLE III. Rare and New Mutations*

Amino acid change	Nucleotide change	Genomic[a] nucleotide no.	cDNA[b] nucleotide no.	Exon	Rapid detection method	Reference
R48W	C → T[c]	1763	259	3		Beutler et al., 1995
R120W	C → T[c]	3059	475	5	–*Msp*I/–*Nci*I	Chabás et al., 1996
I161S	**T → G**	**3393**	**599**	**6**		**First report**
G202R	G → A[c]	3515	721	6	–*Msp*I/–*Nci*I	Beutler et al., 1994
G265D	**G → A**	**4260**	**911**	**7**		**First report**
R285C	C → T	4319	970	7	+*Nsi*I[d]	Beutler et al., 1994
D399N	G → A	5927	1312	9	–*Sal*I/–*Taq*I	Beutler and Gelbart, 1994
F411I	**T → A**	**5963**	**1348**	**9**		**First report**

*Previously undescribed mutations in bold.
[a]Genomic numbering is according to Horowitz et al. [1989].
[b]cDNA numbering starts at the A of the first ATG.
[c]The mutation is present in the normal sequence of the pseudogene.
[d]Mismatched PCR and digestion.

alleles, and mutations Rec*Nci*I and L444P are represented at frequencies of 21% and 6.5% respectively (Fig. 2). The rest of the identified mutant alleles (19.3%) is accounted for by ten different mutations, eight of which were found only once in our patients. Six and one-half percent of the alleles remain unidentified. Three mutations are described here for the first time.

The Rec*Nci*I allele results either from a gene–pseudogene crossing-over leading to a gene fusion or from a gene conversion event [Zimran et al., 1990b]. While ASO hybridization or digestion analysis cannot differentiate between these two mechanisms, Southern blot analysis does: GD complex alleles caused by gene conversion are assumed to present the same band pattern as controls (see Fig. 1b). Five cases of those bearing the Rec*Nci*I allele in Argentinian patients could be explained by a gene conversion event, while an unequal crossing over which produces a fusion gene is responsible for the complex allele (XOVR) identified in Patient A48 [Zimran and Horowitz, 1994]. Unexpectedly, a third pattern of 18–16–12 kb was found in six unrelated patients. These alleles are currently being analyzed to determine the structure and possible mechanism involved.

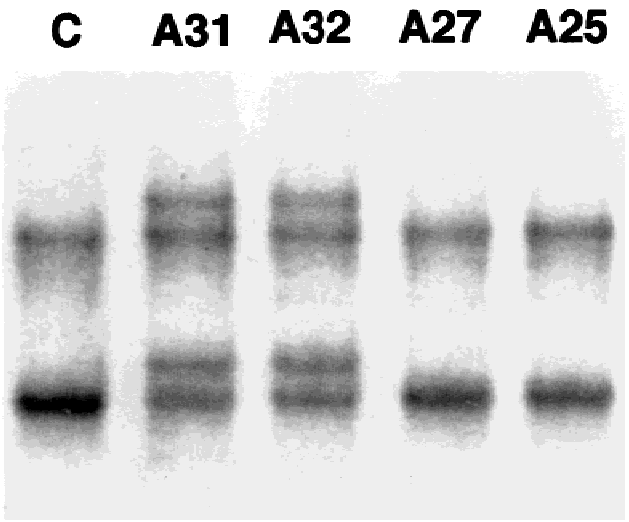


Fig. 3. Detection of the new mutation G265D (911G→A) by SSCP analysis. The PCR-amplified fragment corresponds to exon 7b (3′ region of exon 7). The genotype for the sibs A31 and A32 is N370S/G265D, while those of patients A27 and A25 are N370S/I161S and N370S/D399N, respectively. Mutation I161S is in exon 6 and mutations N370S and D399N in exon 9. C: control, unaffected individual. SSCP conditions: 12% polyacrylamide, electrophoresis at room temperature, 200 V, 16 hr.

The prevalence of Rec*Nci*I is always below 8% in all populations studied to date: It is rare among Ashkenazi Jewish patients [Beutler et al., 1992a; Horowitz et al., 1993], and more frequent in non-Jewish populations, where it ranges from 2.8% in Spanish patients [Cormand et al., 1998] to 7.8% in a mixed group of non-Jewish patients [Horowitz et al., 1993] or 7.2% in Australasian GD patients [Nelson et al., 1995]. It should be mentioned that in some cases Rec*Nci*I alleles could have been erroneously considered as L444P, if the other mutations present in the complex allele were not analyzed.

The three novel mutations described here (I161S, G265D, and F411I) are amino acid substitutions, and several facts suggest that they are disease-causing mutations. First, after exhaustive examination of the glucocerebrosidase gene, no other mutation was found in the patients. Second, the changes were not present in 80 normal chromosomes. Third, all these amino acid residues are conserved in mouse [O'Neill et al., 1989] and human GBA, indicating functional/structural relevance. Finally, two of these substitutions (I161S and G265D) involve a change in the polarity or charge of the amino acid.

Severity of the Phenotypes Caused by Different Mutations

The presence of the N370S allele either in homozygosity or in heterozygosity in 27 GD patients (see Table II) was invariably associated with the Type I phenotype without neurological manifestations, in agreement with previous reports [Beutler and Grabowski, 1995].

In Type I patients classification of mutations by severity is not possible when the allele under study is inherited together with a mild mutation, as is the case for mutations R120W (475C→T), I161S, G202R (721G→A), G265D, R285C (970C→T), or D399N (1312G→A) found together with the common mild N370S mutation. However, previous data could help to classify D399N [Beutler and Gelbart, 1994; Tayebi et

TABLE IV. Homozygosity at the *GBA* Locus as a Measure of Genetic Heterogeneity in Different Populations

Population	$\sum x_i^{2*}$	No. of chromosomes	% Mutation detection	Reference
Norrbottnian	1.00 (1.00)	22	100	Dahl et al., 1990
Ashkenazi Jewish	0.64 (0.65)	1160	89.0	Grabowski., 1997
Ashkenazi Jewish	0.59 (0.59)	276	99.3	Balicki and Beutler, 1995
Ashkenazi Jewish	0.50 (0.51)	354	90.4	Horowitz et al., 1993
Portuguese	0.31 (0.33)	54	87.0	Amaral et al., 1996
Argentinian	0.29 (0.29)	58	93.1	Present study
Spanish	0.24 (0.25)	106	95.3	Cormand et al., 1998
Non–Jewish	0.19 (0.20)	240	89.2	Balicki and Beutler, 1995
Australasian	0.17 (0.25)	56	69.6	Lewis et al., 1994; Nelson et al., 1995
Non–Jewish	0.16 (0.21)	140	77.1	Horowitz et al., 1993

*Uncharacterized alleles were counted as different alleles or as one single allele (the latter shown in parentheses).

al., 1996] and R120W [Latham et al., 1991; Chabás et al., 1996] as severe mutations.

The missense mutations R48W (259C→T) and F411I were found in heterozygosity with the severe mutations Rec*Nci*I and D409H in this study. Previous data suggest that R48W could be a mild mutation [Beutler et al., 1995; Uchiyama et al., 1994]. In Patient A50 from our panel, clinical data are consistent with Type I disease, although given the current age of the individual (5 years) a later neurological development cannot be ruled out. The severity of the newly described F411I mutation cannot be assessed because it is present in an individual (A51) who is only 2 years old.

It should be noted that the genotype/phenotype correlation described above only refers to the classification of the mutations as lethal, severe, or mild as described in Beutler et al. [1994] and that correlations based upon the experience with one or two patients should be taken cautiously due to the considerable phenotypic heterogeneity even in patients sharing the same genotype.

Allelic Heterogeneity

Norrbottnian GD patients all have the same mutation (L444P) [Dahl et al., 1990], resulting in a homozygosity value of 1 (Table IV). Ashkenazi Jews are also very homogeneous (0.50–0.65) due to the high prevalence of mutations N370S and 84GG [Balicki and Beutler, 1995; Horowitz et al., 1993; Grabowski, 1997]. The Argentinian GD population has an intermediate value of 0.29, which is a reflection of both the relatively high frequency of two mutations, N370S and Rec*Nci*I, and the diversity of the remaining GD alleles.

Linkage Disequilibrium Studies

In this study, the N370S allele was always found in the context of the "–" haplotype, while mutation L444P appears associated to both haplotypes. This is consistent with a single origin of N370S mutation and suggests multiple origins for L444P in different populations. These data are in agreement with previous reports for various populations [Zimran et al., 1990a; Beutler et al., 1991, 1992b; Tuteja et al., 1993; Amaral et al., 1996; Cormand et al., 1998].

The high prevalence of the complex allele Rec*Nci*I among Argentinian patients could have been the consequence of a founder effect. This hypothesis was ruled out because it was shown that three different mechanisms (correlated with three different Southern patterns) were involved in the origin of these alleles. However, when grouping the alleles according to Southern patterns, interesting associations arose. All of the alleles presumably derived from a gene conversion event were always found in the context of the less common "+" haplotype, while those alleles that present the previously undescribed 18–16–12 kb pattern are all associated with the "–" haplotype. The data are consistent with the expansion of few ancestral Rec*Nci*I alleles in the Argentinian population. The linkage disequilibrium observed between the alleles presumably derived from gene conversion and the "+" haplotype would back this hypothesis although a larger sample and an extended haplotype analysis using markers flanking the glucocerebrosidase gene [Cormand et al., 1997b] would be required to confirm this association.

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