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A New Gene–Pseudogene Fusion Allele Due to a Recombination in Intron 2 of the Glucocerebrosidase Gene Causes Gaucher Disease

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ABSTRACT: Gaucher disease is the most prevalent sphingolipid storage disorder in humans caused by a recessively inherited deficiency of the enzyme glucocerebrosidase. More than 100 mutations have been described in the glucocerebrosidase gene causing Gaucher disease. Some of them are complex alleles with several mutations due to recombination events between the gene and its highly homologous pseudogene. The generation of these recombinant alleles involves, in most cases, a crossover in the 3′ end of the gene, beyond exon 8. However, in a few cases recombination took place in a more upstream location. Here we describe the analysis of a patient with type I Gaucher disease who bears a new complex allele. This allele was originated by a crossover between the gene and the pseudogene at intron 2, the most upstream recombination site described so far, which gave rise to a fusion gene. The patient was first diagnosed as homozygous for the c.1226 A → G (N370S) mutation but the early onset of the disease prompted us to perform parental DNA analysis which showed that the mother was not a N370S carrier, suggesting deletion of at least part of the gene. Molecular analysis of the complex allele was carried out by Southern blot, PCR, and sequencing. We were able to close down the region of the recombination event to an interval of 18 nucleotides, corresponding to the last 15 nucleotides of intron 2 and the first 3 nucleotides of exon 3 of the gene. These 18 nucleotides are identical between the gene and pseudogene making any further refinement impossible. An exhaustive list of published glucocerebrosidase complex alleles, describing their recombination points, is included for comparison.

INTRODUCTION

Gaucher disease (GD) is an inherited deficiency of the lysosomal enzyme glucocerebrosidase, encoded by the *GBA* gene on chromosome 1p21. Many disease-causing alleles have been described in the *GBA* gene (1) most of them being point mutations. A highly homologous pseudogene, *GBAP*, originated by an ancestral gene duplication, is located 16 kb downstream. The presence of these homologous regions in a small genomic interval enhances the probability of rearrangements resulting in the production of the so-called "complex alleles" or "Rec" mutations. These alleles are generated either by crossovers or gene conversion events between the *GBA* gene and its pseudogene. All of them were found in heterozygosis, suggesting lethality in homozygosis. The few homozygous cases described to date correspond to abortions or neonatal deaths (2–4).

The first gene-pseudogene fusion allele was described in 1990 (5). It bears several sequence changes, normally present in the pseudogene: two missense mutations, L444P and A456P, and the silent polymorphism V460V. Since then, alleles with these changes, usually known as Rec*Nci*I, have been reported in different populations, being the most frequent complex allele in GD. Another complex allele, Rec*TL* (6), which bears the same changes as Rec*Nci*I plus D409H, has also been described several times. Now it is clear that each of these Rec alleles could have arisen from different mutational mechanisms such as gene conversion or unequal crossing over. In most cases

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this issue was not addressed and the name Rec*Nci*I or Rec*TL* is used independently of the mutational mechanism. Besides these two relatively frequent complex mutations, some other recombinant alleles have been reported in individual cases. The recombination site for most of them lies in the 3′ end of the gene, beyond exon 8 (1, 7, 8). Only few crossovers have been described in the 5′ end of the *GBA* gene such as those described by Reissner et al. (9) in intron 3 or Filocamo et al. (10) in intron 6.

Here we report a new recombinant allele, present in heterozygosis in a type I GD patient. An exhaustive molecular characterization of this allele allowed the refinement of the crossover site to an 18-bp interval in the boundary between intron 2 and exon 3 of the *GBA* gene. This is the most upstream recombination event described so far in a GD mutant allele.

MATERIALS AND METHODS

Patient

The patient, now a 14-year-old boy, was clinically diagnosed of Gaucher disease at the age of 2 years 10 months. Diagnosis was confirmed by low glucocerebrosidase activity in cultured fibroblasts. He presented severe splenomegaly from the first months of life but development has been normal despite important splenomegaly (17.5 cm below c.b.) and hepatomegaly. He has no hemorrhagic diathesis. Neurological involvement is absent. Radiologically, the lower end of both femora show the characteristic Erlenmeyer flask appearance. The patient has no bone pain or pathological fractures. Recent analyses show diminished levels of cholesterol and Fe, and normal (in the low range) leukocyte and platelet counts. A bone marrow aspirate showed storage of lipid cells. Clinical data were provided by Dr. T. Toll. (Hospital Sant Joan de Déu, Barcelona, Spain). This patient was partially described as patient I.3 in Cormand et al. (11).

Enzymatic Analyses

The β-glucosidase activity was measured with *N*-stearoyldihydroglucosylceramide (1 mM) or 4-methylumbelliferil-β-glucopyranoside (4.5 mM) in the presence of sodium taurocholate (1.5% w/v) and Triton X-100 (0.2% v/v), as previously reported (12).

DNA Isolation

Genomic DNA was prepared from fibroblasts using the salting out procedure (13).

Sequence Numbering

Nucleotides are numbered throughout the paper according to the recommendations by Beutler and Gelbart (1). The A of the first ATG of the *GBA* gene is considered as nucleotide +1. This implies the subtraction of 583 nucleotides from the genomic sequence by Horowitz et al. (14). This sequence was corrected afterwards (Ref. 15; GenBank Accession No. J03059). However, we have not taken into account this corrected version for nucleotide numbering, in order to allow comparisons with previous published results. This produces small inconsistencies in the length of some of the PCR or Southern fragments.

Detection of the c.1226 A → G (N370S) Mutation

The N370S mutation in exon 9 of the glucocerebrosidase gene was analyzed in the patient through mismatched PCR amplification and *Xho*I digestion as previously described (16). The analysis was also performed on DNA material from the parents and an unaffected brother.

Southern Blot, Probe Preparation, and Hybridization

Genomic DNA was single digested with *Ssp* I, *Bam* HI, and *Xba*I enzymes, 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 (Biotech). Reverse transcription was performed using the Time Saver cDNA Synthesis Kit (Pharmacia Biotech) with an antisense primer at the 3′-UTR end of the gluco-

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cerebrosidase mRNA (5′-CTCTTTAGTCACA-GACAGCG-3′, genomic position 6173–6192). The full coding region of the cDNA was PCR-amplified in a 1836-bp fragment using the reverse transcription primer described above and a sense primer at the 5′ end of the cDNA (5′-GCCG-GAATTACTTGCAGGGC-3′, genomic position −140 to −121). The PCR was performed with the Expand Long Template PCR System (Boehringer Mannheim). The glucocerebrosidase cDNA probe was labeled with [α-^{32}P]dCTP by random priming.

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.

Characterization of the GBA Gene–Pseudogene Fusion by PCR Amplification and Sequencing

A forward gene-specific primer (5′-CCGT-GTTCAGTCTCTCCTAG-3′, genomic position 954-973) in intron 2 of the *GBA* gene and a reverse nondiscriminatory primer (5′-CCT-CAGGGCCTGAAAAAGCT-3′, genomic position 2601–2620) in intron 5 were used to amplify a 1675-bp gene product in normal chromosomes or a 1024-bp gene/pseudogene fusion product in mutated chromosomes. The PCR was performed under the following conditions: 100 ng of template DNA, 1 U of Expand High Fidelity polymerase (Boehringer Mannheim), 200 μM dNTPs, 10 pmol of each primer, in the recommended buffer in a final volume of 25 μl. The PCR program consisted of 35 cycles of denaturation at 94°C for 40 s, annealing at 62°C for 30 s, and extension at 68°C for 2 min.

The PCR product was purified by GFX PCR DNA and Gel Band purification kit (Amersham Pharmacia Biotech) and sequenced by fluorescence dideoxy cycle sequencing (ABI 373A Fluorescent DNA sequencer, Perkin–Elmer).

RESULTS

Biochemical Analysis

Acid β-glucosidase (substrate 4MU-β-glucoside) and β-glucocerebrosidase (*N*-stearoyl-dihydroglucocerebroside) were 9 and 15% of normal values. Chitotriosidase activity was 16313 nmol/h × ml (215-fold increase over control).

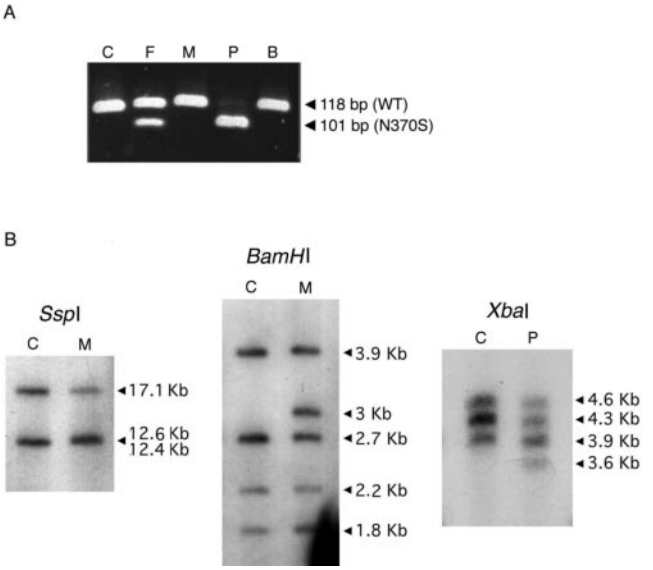

FIG. 1. Characterization of the two mutant alleles of the patient. (A) Detection of mutation N370S by *Xho*I digestion of a mismatched-PCR 118-bp product and electrophoresis on a 2.5% Nu-Sieve agarose gel. The presence of the mutation creates a *Xho*I restriction site, producing digestion fragments of 17 bp (not shown) and 101 bp. C, healthy control; F, father; M, mother; P, patient; B, brother. (B) Southern blot analysis using a 1.8-kb RT-PCR fragment including all the coding region of the *GBA* gene as a probe. Genomic DNA from a healthy control individual (C) and the patient's mother (M) or the patient (P) was single digested with *Ssp*I, *Bam*HI, and *Xba*I. The lower intensity of a 17.1-kb band (*Ssp*I) and the appearance of extra bands of 3 kb (*Bam*HI) and 3.6 kb (*Xba*I) suggest the presence of a rearrangement between the *GBA* gene and its highly homologous pseudogene.

Mutation Characterization

The patient was originally misdiagnosed as homozygous for the common N370S mutation (12) as the PCR-based analysis showed only the pattern corresponding to the N370S allele. However, family data showed that only the father was a N370S carrier (Fig. 1A), suggesting a deletion in the maternal allele.

To characterize this deletion, Southern analyses with enzymes *Ssp*I, *Xba*I, and *Bam*HI were performed. Note that due to limited material from the patient (P), some of the experiments were

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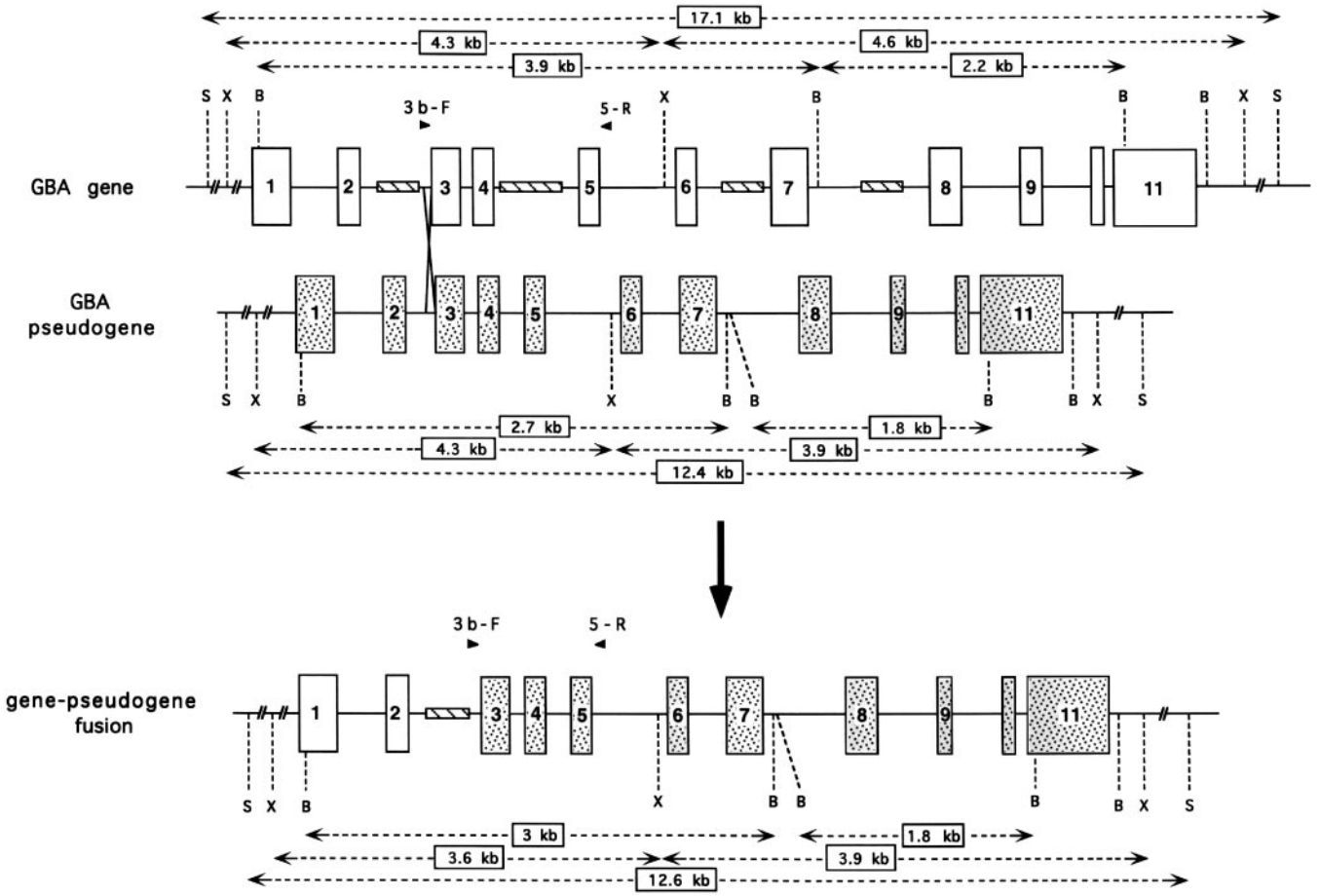

FIG. 2. Schematic representation of the *GBA* gene (open boxes), the *GBA* pseudogene (shadowed boxes) and the gene-pseudogene fusion. The predicted *Ssp*I (S), *Bam*HI (B) and *Xba*I (X) restriction and the expected sizes of the digestion products are shown. *Alu* sequences within the gene are depicted as hatched boxes. The location of PCR primers 3b-F (specific for the gene) and 5-R (nonspecific) used in Fig. 3 and the crossover site are indicated.

performed using maternal DNA (M), which has the same deletion as the patient. *Ssp*I digestion (Fig. 1B) showed a two-band pattern of about 17 (corresponding to the gene) and 12 kb (corresponding to the pseudogene), similar to that of the wild type (Fig. 2). The lower intensity of the upper band suggested the complete deletion of the gene. However, digestion analysis with other enzymes, such as *Bam*HI and *Xba*I, showed different patterns when compared to control DNA. In particular for *Bam*HI, an extra band of approximately 3 kb was obtained, while an extra band of 3.6 kb was found with *Xba*I (Fig. 1B). Moreover, some of the bands, such as the *Xba*I 3.9 kb band in the patient DNA, are more intense than others. These results suggest that a genomic rearrangement, different from a complete deletion of the gene, should have taken place.

The data are consistent with a crossover occurring between the first and the second *Alu* sequences of the gene and its homologous region in the pseudogene. The size of the bands corresponding to the normal and rearranged *GBA* genomic region digested with *Ssp*I, *Bam*HI, and *Xba*I are indicated in Fig. 2. Note that the *Ssp*I fragment containing the fusion gene has a similar, but not identical, size to that of the wild type pseudogene fragment. For the exact length of the fragments, distances between restriction sites were calculated from the published 75-kb sequence of the *GBA* region (GenBank Accession No. AF023268; Ref. 17).

We performed a PCR experiment in order to confirm the hypothesis that the gene-pseudogene crossover occurred in a site located between introns 2 and 4 of the gene. While the forward

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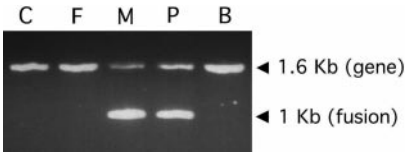

FIG. 3. Characterization of the *GBA* gene-pseudogene fusion by PCR amplification. The location of primers is indicated in Fig. 2. A 1-kb fusion band was obtained from the mother (M) and the patient (P), whereas the father (F) and an unaffected brother (B) only showed the 1.6-kb gene band. A healthy control individual (C) was included.

primer (intron 2) is specific for the gene, the reverse primer (intron 5) is not specific. Amplification on control genomic DNA produces a 1.6-kb fragment from the gene, and no product from the pseudogene. If the hypothesis is correct, a 1.0-kb band should be produced from the fusion gene. Figure 3 shows that both the patient and his mother bear this fusion product, whereas the father and the brother do not.

Upon cloning and sequencing the fusion product, a precise localization of the crossover was obtained. As shown in Fig. 4, the recombinant allele bears an "A" at position 1021 (as in the normal gene sequence) and a dinucleotide "GT," present in the pseudogene, instead of a "CCC" trinucleotide at positions 1040–1042 of the gene. All downstream sequences belong to the pseudogene. These data close down the point of the crossover to nucleotides 1022–1039. The fact that this 18-nt region shows no differences between gene and pseudogene precludes any further refinement.

DISCUSSION

Several complex alleles of the glucocerebrosidase gene have been described. The two most common alleles, Rec*Nci*I and Rec*TL*, are identified according to the pseudogene variants they bear. However, this classification does not take into account neither the actual site of the recombination nor the molecular mechanisms by which these alleles were generated. Although Southern blot analyses or long-PCR amplification could help to understand the mechanism underlying these mutational events, only in a few cases has this issue been addressed (for example, Refs. 5, 9, 18). Most of the reported complex alleles involve a crossover in the 3′ region of the gene, beyond exon 8 (1, 7, 8). As suggested, recombinational hotspots could be present in this region. However, it is also possible that, due to a technical bias, some of the crossovers occurring farther upstream could be missed. Usually, the presence of recombinant alleles is only investigated when L444P, a mutation normally present at the 3′ end of the pseudogene, has been detected. However, as the usual detection protocol for this mutation involves digestion of a PCR-amplified fragment, it could be missed in alleles bearing pseudogene sequences in the annealing site for the gene-specific primers. To our knowledge, the most upstream crossover described so far was located in intron 3 (9). Here we describe a new complex allele which is due to a recombinant event in a more upstream position, in particular between intron 2/exon 3 of the gene and the homologous site of the pseudogene.

The patient was originally diagnosed as an N370S homozygote (12). This misdiagnosis was due to the lack of amplification of the maternal allele. Similar cases have been also described (19, 20). In all these situations, the severe symptoms presented by the patient or the early onset of the disease prompted a more detailed analysis which allowed correction of the original genotype determination. The combination of Southern blot anal-

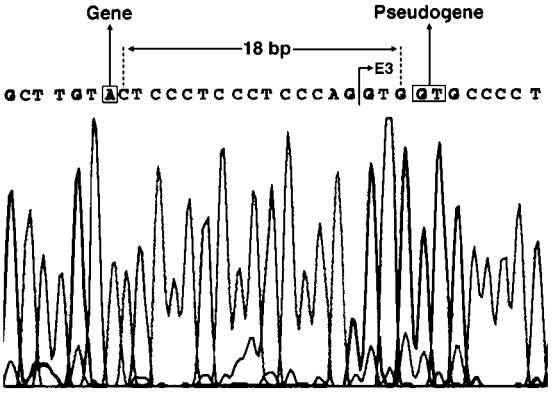

FIG. 4. Determination of the *GBA* gene-pseudogene crossover site by sequence analysis. The 1-kb PCR product shown in Fig. 3 was sequenced using primer 3b-F, which recognizes a specific sequence in intron 2 of the *GBA* gene. The position of the crossover was narrowed down to a genomic interval of 18 bp between a gene-specific nucleotide (A) and pseudogene-specific nucleotides (GT).

TABLE 1

Complex Alleles Reported to Date

First crossover[a]	Second crossover[b]	Exons affected	Allele names	Pseudogene changes in the fusion (or converted) gene[c]	References
1022–1039 (int 2–ex 3)	—	3–11	Rec (int-2)	33 mutations 13 polymorphisms	This study
Intron 3	—	4–11	Rec A	19 mutations 8 polymorphisms	(7, 9)
2456–2476 (int 4–ex 5)	2965–3578 (ex 6–int 6)	5–6	Complex C	R120W, W184R, N188K, V191G, S196P, G202R, F213I	(24)
Intron 6	—	7–11	Complex I	7 mutations 5 polymorphisms	(10)
Intron 8–exon 9	—	9–11	Rec B	del55, D409H, L444P, A456P, V460V	(7)
Intron 8–exon 9	—	9–11	Rec(g4889–6506)	del55, D409H, L444P, A456P, V460V	(25)
5005–5294 (int 8–ex 9)	5690–5722 (int 9)	9	Rec[1263del55, 1342G>C]	del55, D409H, L444P, A456P, V460V	(26)
5005–5294 (int 8–ex 9)	5900–6731 (ex 10–ex 11)	9–10/11	c1263del+Rec*TL*	del55, D409H, L444P, A456P, V460V	(27)
5349–5373 (ex 9)	—	9–11	Rec*TL* Complex B	D409H, L444P, A456P, V460V	(6, 28)
Exon 9	—	9–11	Rec C	D409H, L444P, A456P, V460V	(7)
5374–5689 (ex 9–int 9)	5886–5898 (ex 10)	9–10	RecA456P	D409H, L444P, A456P	(27)
5374–5689 (ex 9–int 9)	—	10–11	Rec*Nci*I Complex A	L444P, A456P, V460V	(5, 6, 28)
Intron 9	—	10–11	Rec D	L444P, A456P, V460V	(7)
5689–5723 (int 9)	—	10–11	Rec allele of patient 1043	L444P, A456P, V460V	(29)
Intron 9	—	10–11	Rec E	L444P, A456P, V460V	(7)
Intron 9–exon 10	—	10–11	Rec F	L444P, A456P, V460V	(7)

Note. int, intron; ex, exon.

[a] Location of first crossover or 5′ limit of gene-converted region.

[b] Location of second crossover or 3′ limit of gene-converted region. (—) Indicates nonexistant or not described crossover.

[c] Only mutations in the coding sequence or splice sites were considered.

ysis, PCR amplification and sequencing allowed the complete characterization of this mutant: it is a recombinant allele with the crossover point located within a 18-bp interval at the end of intron 2 and beginning of exon 3. This allele, which we have named Rec(int-2), is a null allele, as it includes a frameshift mutation early in exon 3 (g.1040delC). All the band sizes observed in Southern analyses are consistent with this crossover site according to the restriction site data within the 75-kb published sequence (17). However, the higher intensity of the 4.3 kb band obtained after control DNA digestion with *Xba*I (see Fig. 1B) suggests the existence of an extra *Xba*I site, 5′ of the pseudogene, not present in the reported sequence. We propose that a sequencing error or a polymorphism could have occurred in the nucleotide 52090 of the 75-kb sequence, converting a C to a G and the sequence "TCTA**C** A" (nt. 52086–52091) to "TCTA**G** A," a *Xba*I site. This would generate a 4248-bp fragment that would comigrate with the 4257 bp fragment of the gene (see Fig. 2).

Large deletions are difficult to characterize. Particularly, it may be complicated to differentiate a fusion gene generated by a crossover in the 5′ region of the gene from a complete deletion of the gene, as a PCR amplification of part of the gene could fail to make this distinction, depending on the primers and the site of the crossover. Moreover, the *Ssp*I analysis, frequently used to detect rearrangements, could also be non-informative, as shown in the case presented in this work (Fig. 1B).

Several complex alleles have been published so far. Most of them are listed in Table 1, not including those reported only in abstracts or con-

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gress communications. In some cases, an internal part of the gene has been replaced by the homologous sequence of the pseudogene. Both gene conversion or double crossover events have been suggested as putative mutational mechanisms. The two crossover sites (or the two limits of the converted region) are indicated for them in Table 1. On the other hand, complex alleles presenting gene sequences at their 5′ end and pseudogene sequences at the 3′ end, may derive from a single crossover event. They are consistent with the generation of a gene-pseudogene fusion resulting in the deletion of the chromosomal material between them. Characterization of this kind of allele may be difficult and it has seldom been performed. In some cases the recombination site has not been precisely identified. Thus, some of the complex alleles found in independent patients and bearing the same combination of pseudogene mutations could represent indeed the same allele. In the case presented here we were able to confirm the generation of a fusion gene and to define the crossover region precisely in the 5′ end of the *GBA* gene.

An increasing number of reports suggest that recombinant alleles are a frequent cause of mutation in GD (18, 21–23), and also a possible source of genotyping misdiagnosis (11, 19, 20). The incorrect assignment of mutated alleles may eventually lead to confusing clinical correlations, and further complicate the prognosis of the disease. A focused search and complete characterization of complex alleles, including Southern blot and long PCR methods, would shed light both on mutation mechanisms and genotype-phenotype correlations in Gaucher disease.

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