Anàlisi molecular de la mucopolisacaridosi I, la mucopolisacaridosi II i la leucodistròfia metacromàtica en els pacients espanyols. Utilitat diagnòstica i correlació genotip-fenotip

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DISCUSSIÓ GENERAL

1. ANÀLISI MOLECULAR I LA SEVA UTILITAT DIAGNÒSTICA

En aquest treball s'ha fet l'anàlisi molecular de tres de les malalties lisosòmiques més freqüents en la població espanyola, la mucopolisacaridosi de tipus I, la mucopolisacaridosi de tipus II i la leucodistròfia metacromàtica. L'objectiu d'aquest estudi ha estat observar l'espectre de mutacions de cada malaltia per poder establir la millor estratègia de diagnòstic molecular de cara als pacients i als seus familiars.

En estudiar aquestes tres malalties, ens hem trobat amb tres casos ben representatius de com pot ser l'espectre mutacional d'una malaltia hereditària:

- Dues o tres mutacions són les principals causants de la malaltia, per tant, l'estudi d'aquestes cobreix la immensa majoria dels al·lels (més del 70%), i la resta poden presentar tot tipus de mutacions. Aquest és el cas de la **mucopolisacaridosi de tipus I**, en la qual les principals causants de la malaltia en la població espanyola són les tres mutacions W402X, Q70X i P533R.
- Dues o tres mutacions es troben en percentatges similars respecte el total d'al·lels per la malaltia als pacients que la presenten, l'anàlisi de totes aquestes mutacions juntes arriba a cobrir la meitat o poc més dels al·lels i la resta tenen un caràcter molt heterogeni. Aquest és el cas de la **leucodistròfia metacromàtica**, que amb l'estudi de tres mutacions diferents es cobreix el 55.5% dels al·lels, però per arribar al 72.5% cal l'estudi de set mutacions diferents.
- No hi ha cap mutació que sigui especialment majoritària en la població de malalts, sinó que hi ha una gran heterogeneïtat genètica i gairebé cada pacient presenta una mutació diferent i particular; si alguna mutació es troba en més d'una família, no arriba mai a percentatges significatius. Aquest és el cas de la **mucopolisacaridosi de tipus II**, que es comporta com una malaltia amb una gran heterogeneïtat genètica en la qual les mutacions més prevalents són les grans delecions i reordenaments, que arriben a representar l'11% dels al·lels per la malaltia.

Podem veure, doncs, que tenim tres exemples que representen els dos extrems i un pas intermedi del ventall d'espectres de mutacions que es poden trobar en les malalties hereditàries. Així mateix, els resultats obtinguts en cada malaltia fan

plantejar diferents estratègies pel diagnòstic molecular de cara als propers pacients que es diagnostiquin bioquímicament.

Per una malaltia com la mucopolisacaridosi de tipus I, que amb tres mutacions es cobreix la immensa majoria dels al·lels, evidentment, la millor estratègia davant un nou pacient per diagnosticar, és analitzar aquestes tres mutacions ja que probablement es cobrirà el genotip, i el diagnòstic de portadors i el diagnòstic prenatal als seus familiars no oferirà cap problema. Si no s'identifica algun dels al·lels, es pot renunciar a la identificació de la mutació concreta en aquest al·lel i realitzar el diagnòstic dels familiars mitjançant l'estudi indirecte per marcadors polimòrfics. En aquesta malaltia en concret, aquesta estratègia és la més recomanable, ja que la presència d'una trentena de marcadors polimòrfics faciliten aquest estudi i, en canvi, dificulten l'aplicació de tècniques de recerca de mutacions com ara l'SSCP.

Per una malaltia com la leucodistròfia metacromàtica, que amb set mutacions es cobreix més del 70% dels al·lels, la millor estratègia a seguir pel diagnòstic molecular dels nous casos, és l'estudi d'aquestes mutacions començant per les més freqüents. Probablement, s'identificaran la major part dels al·lels i en aquests casos es podrà realitzar l'estudi de portadors i el diagnòstic prenatal amb absoluta fiabilitat. Per caracteritzar els al·lels que no portin aquestes mutacions es pot optar per fer una recerca de les mutacions per mètodes com l'SSCP, o bé, i potser és el més recomanable, realitzar l'estudi familiar per estudi indirecte amb marcadors polimòrfics i deixar la mutació sense identificar.

Per una malaltia com la mucopolisacaridosi de tipus II, la gran heterogeneïtat genètica indica que la millor estratègia a seguir per poder oferir el diagnòstic familiar amb certa rapidesa, és fer directament la caracterització de l'haplotip associat a l'al·lel de la malaltia en el nen afecte per estudi de marcadors polimòrfics intra i/o extragènics, i fer el diagnòstic de portadores i el prenatal per estudi indirecte. Indubtablement, sempre és millor el diagnòstic per identificació directe de la mutació, però en aquesta malaltia això representa una gran despesa de temps i recursos, a considerar tant per les famílies com pels centres de diagnòstic.

Així doncs, els resultats obtinguts de l'anàlisi molecular d'aquestes tres malalties lisosòmiques, ens han dut a establir tres estratègies de diagnòstic diferents i depenent de les característiques de l'espectre de mutacions detectat per cada malaltia en la població espanyola afecta.

Deixant de banda la mucopolisacaridosi de tipus II, ja que la seva gran heterogeneïtat genètica li confereix unes característiques particulars que no permeten gaires comparacions, els estudis realitzats en la mucopolisacaridosi de tipus I i en la leucodistròfia metacromàtica han fet observar que la població espanyola presenta unes característiques genètiques molt pròpies. En ambdós casos, les mutacions que es presenten en major percentatge també s'han detectat en altres poblacions però en l'espanyola es presenten en un percentatge més elevat. Aquest és el cas de la mutació W402X en la MPS I, i les mutacions IVS2+1G→A, i D255H en la leucodistròfia metacromàtica.

Una observació similar s'ha fet en la malaltia de Gaucher, en què les mutacions N370S i L444P es presenten en un percentatge molt elevat en pacients espanyols; amb l'estudi d'aquestes dues mutacions es cobreix un 70% dels al·lels Gaucher, aportant la mutació N370S un 44.3% d'aquests al·lels. Aquesta mutació també s'ha descrit en altres poblacions, però, exceptuant en els jueus asquenasites i en els portuguesos, és en l'espanyola on s'ha trobat una major prevalència d'aquesta (Cormand i col, 1995).

Així mateix, la mutació 1091delC és molt freqüent en els malalts espanyols amb mucopolisacaridosi de tipus IIIA (o malaltia de Sanfilippo A) comparat amb les prevalències descrites en altres poblacions (Montfort i col, 1998).

També en la gangliosidosi GM2 s'han identificat dues mutacions molt freqüents, la R178H i la IVS4+5G→A. Ambdues mutacions han estat descrites anteriorment, la R178H s'ha identificat amb certa prevalència en altres poblacions, mentre que la mutació IVS4+5G→A només s'ha identificat en un parell de casos fins ara. En la població espanyola, en canvi, amb l'estudi de les mutacions R178H i IVS4+5G→A es cobreix un 26% i un 35% dels al·lels GM2, respectivament, el que representa que només amb l'estudi d'aquests dos canvis es poden identificar fins a un 61% dels al·lels per la malaltia (Gort i col, manuscrit en preparació).

Aquestes dades ens indiquen que, quan es vol abordar un estudi molecular de les malalties lisosòmiques en la població espanyola, en general, es podrà establir una estratègia de detecció de certes mutacions que permetran tipificar un alt percentatge dels al·lels per la malaltia, el que facilitarà enormement el diagnòstic tant de pacients com dels familiars que ho sol·licitin. Caldran, però, més estudis per esbrinar el perquè d'aquestes característiques genètiques de la nostra població.

2. ESTUDI D'HAPLOTIPS I ORIGEN DE LES MUTACIONS

Independentment de que es posi a punt l'estudi d'haplotips en cada una de les tres malalties mencionades per poder fer el diagnòstic de portadors i el diagnòstic prenatal per estudi indirecte, aquest anàlisi també és útil per veure si una mutació és recurrent o bé té un origen únic. Aquest tipus d'estudis només tenen sentit si hi ha una mutació que es presenta amb certa freqüència en una població i es poden comparar els haplotips que presenta cada al·lel en un nombre suficientment significatiu. És per això, que ja d'entrada, en una malaltia amb un espectre de mutacions amb les característiques del de la mucopolisacaridosi de tipus II, aquest tipus d'estudis no es poden realitzar, ja que encara que hi hagi una mutació que la presenti més d'un pacient, el nombre és tan reduït que no se'n pot treure cap conclusió clara.

Les característiques mutacionals de la mucopolisacaridosi de tipus I i de la leucodistròfia metacromàtica sí que fan que un estudi d'haplotips per intentar establir el tipus d'origen de les mutacions freqüents tingui sentit.

Els resultats obtinguts en aquest estudi indiquen que ens trobem davant dues malalties de naturalesa diferent: mentre que en la MPS I la mutació més freqüent, la W402X, sembla ser deguda a una gran recurrència, ja que s'ha trobat associada a diferents haplotips, en la leucodistròfia metacromàtica la mutació més freqüent, la IVS2+1G→A, està sempre associada a un mateix haplotip, el que indica que probablement té un origen únic.

L'estudi de les altres mutacions freqüents de la LDM, la D255H i la T327I, han demostrat que, als pacients espanyols, també estan associades a només un haplotip, el que suggereix que les mutacions es van originar cada una, en un moment determinat de la història, en un al·lel amb un determinat haplotip, i que l'alta freqüència en la població de malalts és deguda a l'expansió d'aquest al·lel inicial. Això indica que en el gen *ARSA* les mutacions no acostumen a ser recurrents, sinó que aquestes ocorren en un determinat moment i a partir d'aleshores es transmeten per herència.

La MPS I, en canvi, representa l'expressió d'un gen, l'*IDUA*, que té facilitat per ser mutat en uns codons concrets. En aquesta malaltia, doncs, les tres mutacions que es presenten amb més freqüència en la població espanyola, presenten una alta recurrència.

3. CORRELACIÓ GENOTIP-FENOTIP

En totes les malalties lisosòmiques, un dels principals objectius quan es fa l'anàlisi molecular de la malaltia, és intentar establir una correlació entre el genotip i l'expressió clínica. En cas d'existir una correlació d'aquest tipus, aquesta ens permetrà, encara que de forma aproximada, predir l'evolució del pacient i aconsellar amb més encert l'aplicació de possibles teràpies, com ara el trasplantament de moll d'os.

Per poder establir una correlació genotip-fenotip, cal que es disposi d'un nombre significatiu de pacients amb el mateix genotip i amb la clínica clarament caracteritzada. És per això que, igual que en l'estudi d'haplotips, en una malaltia de les característiques mutacionals de la mucopolisacaridosi de tipus II, no es pot establir aquest tipus de correlació per la manca d'un nombre suficient de pacients amb la mateixa mutació, i això que, en tractar-se d'una malaltia lligada al cromosoma X, el pacient representa ja de per si mateix, l'expressió de la mutació, i la correlació mutació-clínica seria més senzilla de trobar. Tot i així, en les mutacions que s'han identificat en més d'un pacient, s'ha intentat fer alguna comparació entre les presentacions clíniques dels pacients que les presentaven, i en tots els casos se n'ha tret alguna petita conclusió, però el reduït nombre de pacients comparat, fa que aquestes conclusions no puguin ser postulades amb la total esperança de que siguin certes. D'altra banda, no té massa sentit intentar extreure conclusions sobre una correlació genotip-fenotip d'unes mutacions que es troben en molt poca freqüència en la població de malalts, ja que no es podran utilitzar pels objectius pels quals es realitzen aquest tipus d'estudis.

En el cas de la MPS I i de la leucodistròfia metacromàtica, el fet de disposar d'un elevat nombre de pacients que presenten les mateixes mutacions permet establir amb més fiabilitat algun tipus de correlació. En ambdós casos, però, com que es tracta de malalties autosòmiques recessives, cal basar-se sobretot en els pacients que són homozigots per les mutacions, per no veure emmascarada l'expressió clínica d'una mutació, ja que quan es troben en heterozigosi l'efecte que pot produir la mutació de l'altre cromosoma pot alterar molt la presentació de la malaltia en el pacient. Així, s'intenta comparar primerament els pacients homozigots per la mutació, i un cop observades les clíniques i la possible correlació, es recorre als heterozigots per les mutacions estudiades per comprovar si la combinació de les

mutacions es reflecteix en el pacient en forma d'una combinació de les clíniques que dóna cada mutació per separat.

En totes dues malalties, s'ha pogut trobar una clara correlació genotip-fenotip entre les tres (W402X, Q70X i P533R) per la MPS I, i les dues (IVS2+1G→A i D255H), per la leucodistròfia metacromàtica, mutacions més freqüents als pacients espanyols. Aquests resultats creiem que seran de gran ajut de cara a l'assessorament de les famílies amb nous membres afectes que presentin aquests mutacions i de cara al consell genètic. No obstant, s'ha de tenir en compte que altres factors genètics o no també poden influir en l'expressió clínica definitiva de la malaltia.

CONCLUSIONS

De l'estudi realitzat en la mucopolisacaridosi de tipus I, la mucopolisacaridosi de tipus II i en la leucodistròfia metacromàtica, en podem extreure les següents conclusions:

1. Les tres malalties estudiades presenten un espectre de mutacions de característiques absolutament diferents. Aquests espectres mutacionals van des de l'alta homogeneïtat genètica que presenta la MPS I a la gran heterogeneïtat genètica de la MPS II, passant per un punt intermedi representat per la leucodistròfia metacromàtica.

2. En la **Mucopolisacaridosi de tipus I** o malaltia de Hurler/Scheie, l'estudi de les mutacions W402X, Q70X i P533R en els pacients MPS I ha permès identificar el 76% dels al·lels i el 63% dels genotips, resultats que representen una gran avantatge de cara al diagnòstic d'aquesta malaltia en la població espanyola, ja que només amb l'estudi d'aquestes tres mutacions es pot identificar la majoria dels al·lels MPS I, el que representa un estalvi de temps i recursos i una bona eina per al consell genètic dels familiars.

3. La mutació W402X presenta en la població espanyola afecta la freqüència més alta detectada fins al moment per aquesta mutació respecte les altres poblacions estudiades (aprox. un 60%).

4. La demostració que mutacions que són altament freqüents com la W402X estiguin associades a més d'un haplotip indiquen que, o bé la mutació s'ha originat vàries vegades o bé que s'ha originat un sol cop però que hi ha un alt índex de recombinació. Si la mutació s'ha originat vàries vegades indicaria una alta recurrència de la mutació.

5. L'alta freqüència observada per aquesta mutació en la nostra població, l'escassa presència als països nòrdics i unes freqüències intermèdies a centre-europa ens fan postular l'existència d'una genoclina ascendent de la freqüència de la mutació W402X des dels països escandinaus i Rússia cap a la península ibèrica.

6. La freqüència obtinguda per la mutació Q70X en la població espanyola recolza la hipòtesi de l'existència d'una genoclina descendent de la freqüència d'aquesta mutació des dels països nòrdics i Rússia cap al sud d'Europa.

7. La presència de la mutació P533R amb certa freqüència en les poblacions properes al mar Mediterrani com l'espanyola, la italiana, la francesa o la nord-africana, i la quasi absència d'aquesta en la resta de poblacions, indiquen que la mutació P533R és una mutació eminentment mediterrània. L'alta freqüència en població nord-africana fa postular un origen africà d'aquesta mutació, que s'hauria escampat a la resta de poblacions degut als continus contactes al llarg dels segles que els pobladors d'aquest continent han mantingut amb els països que volten el Mar Mediterrani.

8. Les mutacions W402X, Q70X i P533R estan clarament associades a la forma severa de la malaltia, correlació que permet fer un pronòstic del curs de la malaltia en cada pacient i assessorar sobre les possibles teràpies a aplicar.

9. El fet que la mutació P533R es presenti amb una forma més lleu de la malaltia en la població nord-africana que en l'espanyola pot ser degut a un rerafons genètic diferent, amb l'associació a uns haplotips diferents dels que ho està en població espanyola i que aquests puguin modular l'efecte sever de la mutació, suavitzant-lo.

10. Respecte la **Mucopolisacaridosi de tipus II** o malaltia de Hunter, en la població espanyola afectada, existeix una gran heterogeneïtat genètica, reflectida en el fet que un 11% dels pacients presenten grans delecions i reordenaments, un percentatge lleugerament inferior al descrit en malalts d'altres poblacions. Aquest tipus de mutacions, com a bloc són les que hem trobat més sovint en els pacients espanyols. En la resta dels 32 pacients, però, s'han identificat 26 mutacions diferents i el 69% d'aquests pacients presenten una mutació "particular" no descrita en cap altra família.

11. La tècnica de l'SSCP, ha presentat un 90% d'eficàcia per la detecció de les mutacions puntuals al gen *IDS*, un valor superior al descrit per altres grups per aquesta tècnica, i es presenta com un mètode bastant acceptable per la recerca i identificació de canvis al genoma.

12. La distribució de les mutacions a la zona codificant del gen *IDS* als malalts espanyols és coincident amb la descrita fins al moment a les altres poblacions estudiades, essent els exons VIII i IX els que presenten més mutacions, encara que es desconeix la funcionalitat concreta dels dominis que codifiquen.

13. La gran heterogeneïtat genètica observada, dificulta el poder establir una correlació genotip-fenotip en els pacients amb aquesta malaltia, ja que no hi ha gaires mutacions que es presentin en varis pacients com per poder comparar la clínica. Els estudis de correlació genotip-fenotip ajuden a establir el pronòstic del curs de la malaltia a cada pacient i aconsellar la possible teràpia a seguir. Tot i així, s'ha pogut observar que les grans delecions i reordenaments del gen *IDS* estan associats a la forma severa de la malaltia. D'altra banda, la mutació puntual d'*splicing* G374G i la mutació sense sentit R443X, presents en tres pacients espanyols cada una, estan associades a la forma lleu o intermèdia de la malaltia de Hunter.

14. El canvi R313C s'ha demostrat que és una variant no patogènica rara i esdevé així el segon polimorfisme descrit en zona codificant del gen *IDS*.

15. El percentatge similar de dones portadores i de no portadores diagnosticades en l'estudi de les familiars dels pacients espanyols, indiquen que en aquesta població no hi ha selecció positiva per l'al·lel mutat. Els diagnòstics prenatals realitzats, tot i ser pocs casos, recolzen aquesta mateixa observació.

16. Respecte la **Leucodistròfia metacromàtica**, les mutacions més freqüents en la població espanyola de pacients LDM són la IVS2+1G→A, la D255H i la T327I. Amb totes elles es cobreix un 52.5% dels al·lels i un 12.5% dels genotips. Però si afegim, a més, les quatre mutacions L68P, G99V, P377L i P426L, aquest percentatge augmenta fins el 73% dels al·lels.

17. Les dotze mutacions que no havien estat descrites anteriorment i identificades en la població espanyola augmenten fins quasi la norantena el nombre de mutacions diferents descrites al gen *ARSA*.

18. Existeix una correlació clara entre l'activitat enzimàtica de l'arilsulfatasa A i la presentació clínica, sobretot per diferenciar els pacients amb forma infantil dels que presenten formes juvenil o adulta. Tant la mutació IVS2+1G→A com la D255H estan associades a la clínica infantil de la malaltia. Fins ara una associació d'aquest tipus sols s'havia establert amb les mutacions IVS2+1G→A i P426L. Les dades en pacients espanyols han permès afegir la mutació D255H al grup de mutacions amb una correlació genotip-fenotip clara.

19. La freqüència de la mutació polyA- de l'al·lel ARSApd en població control espanyola s'ha establert en un 10.8%. La mutació N350S s'ha trobat, a més, en un 6% dels al·lels controls no associada a la mutació polyA-. S'ha identificat dos individus sans que presenten la mutació del polyA- de l'ARSApd no associada a la mutació N350S. Això indica que el desequilibri de lligament entre la polyA- i la N350S no és perfecte.

20. L'anàlisi molecular per diferenciar la pseudo-deficiència de l'ARSA de la deficiència patogènica de la leucodistròfia metacromàtica és de gran ajut a l'hora de distingir entre pacients, portadors sans de la malaltia i portadors de la pseudo-deficiència, diagnòstic que resulta molt difícil de realitzar només tenint en compte les dades bioquímiques.

21. En la població control s'han identificat quatre haplotips diferents deguts a la combinació dels al·lels de quatre polimorfismes diferents. El fet que manquen algunes de les combinacions indicaria que els marcadors no són independents i que els haplotips que s'han detectat deriven d'un ancestre comú. Les mutacions IVS2+1G→A, D255H i T327I estan en desequilibri de lligament amb certs haplotips, resultats que indiquen un origen únic d'aquestes mutacions.

22. Si tenim en compte **totes tres malalties**, l'estudi d'haplotips en les mutacions més freqüents de la mucopolisacaridosi de tipus I i de la leucodistròfia metacromàtica, han mostrat que, mentre que la MPS I acostuma a ser deguda a unes mutacions que ocorren amb certa recurrència, la leucodistròfia metacromàtica es caracteritza per una relativament alta freqüència de mutacions que tenen un origen únic i que es van expandir en la població.

23. L'existència d'una gran heterogeneïtat genètica en malalties com la MPS II, fan que en aquests casos no es puguin realitzar estudis ni d'haplotips per estudiar l'origen de les mutacions ni de correlació entre el genotip i el fenotip, que deixen de tenir sentit degut a la poca recurrència de les mutacions.

24. Finalment, la millor estratègia pel diagnòstic molecular d'aquestes malalties hereditàries, és fer l'anàlisi de les mutacions que s'han trobat més freqüents en la població espanyola, sobretot si aquestes representen un percentatge significatiu dels al·lels per la malaltia (MPS I i LDM). Pels al·lels que no quedin identificats després d'aquest primer anàlisi, la millor estratègia és caracteritzar-los l'haplotip mitjançant l'estudi de marcadors polimòrfics, d'aquesta manera es podrà oferir als familiars el diagnòstic de portadors i el diagnòstic prenatal per estudi indirecte amb una certa rapidesa. En la mucopolisacaridosi de tipus II, la gran heterogeneïtat genètica fa aconsellable fer el diagnòstic de portadores per anàlisi indirecte per estudi de cosegregació de marcadors polimòrfics.

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Annex

Durant la realització d'aquest treball, també he col·laborat en l'estudi de la malaltia de Gaucher. Concretament, la col·laboració ha estat en la detecció de les mutacions més freqüents en aquesta malaltia: N370S, L444P, D409H, 1263del55, G377S i N396T. En tractar-se d'una tasca bàsicament de diagnòstic no s'ha inclòs en la tesi, però degut a que els resultats han donat lloc a diverses publicacions, s'ha cregut oportú afegir aquests articles com a un annex perquè quedi constància de la feina realitzada.

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Two New Mild Homozygous Mutations in Gaucher Disease Patients: Clinical Signs and Biochemical Analyses

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Gaucher disease (GD) is a lysosomal storage disorder resulting from impaired activity of lysosomal β-glucocerebrosidase. More than 60 mutations have been described in the GBA gene. They have been classified as lethal, severe, and mild on the basis of the corresponding phenotype. The fact that most GD patients are compound heterozygous and that most type 1 patients bear the N370S allele, which by itself causes a mild phenotype, make it difficult to correlate the clinical signs with the mutations. Besides N370S, about 10 mild mutations have been described, but only one undoubtedly classified as mild was found at homozygosity. Here we report 2 novel mutations, I402T and V375L, at homozygosity in 2 adult Italian type 1 GD patients. Some properties of the I402T fibroblast enzyme have been compared to those of the enzyme from cells of several N370S/N370S patients. Analysis of the catalytic properties and heat stability as well as the response to phosphatidylserine and sphingolipid activator protein indicate a marked similarity between the 2 enzymes. The finding of another, unrelated patient bearing the I402T mutation (in this case as a compound heterozygote with mutation N370S) suggests that this allele might be quite frequent in the area of Sicily from where both patients originated. In conclusion, the phenotypic expression in the 2 homozygous patients presented here and the biochemical data for one of them allowed the classification of these mutations as mild thus extending the group of mild mutations found at homozygosity. Am. J. Med. Genet. 70:437–443, 1997. © 1997 Wiley-Liss, Inc.

KEY WORDS: Gaucher disease; mutant glucocerebrosidase; mild mutations; genotype-phenotype correlation

INTRODUCTION

Gaucher disease (GD) is a lysosomal glycolipid storage disorder characterized by the accumulation of glucosylceramide mainly in macrophages. It is common among Ashkenazi Jews [Beutler and Grabowski, 1995]. There are 3 clinical types of GD: type 1, characterized by hepatosplenomegaly, anemia, thrombocytopenia and bone lesions, and by the lack of primary nervous system involvement; type 2, the acute neuronopathic form of the disease, with severe nervous system involvement and death usually within the first 2 years of life; and type 3, the juvenile subacute neuronopathic form, with later onset and a more protracted course than type 2.

Nearly all GD cases are due to mutations in the gene encoding β-glucocerebrosidase (GBA); to date more than 60 mutations have been described [Balicki and Beutler, 1995]. Although genotype-phenotype correlations are difficult to establish, Beutler et al. [1994] classified the mutations on the basis of the severity of the phenotypic expression. Lethal mutations prevent the formation of any enzyme. They have never been found either at homozygosity or as combined heterozygotes with another lethal mutation. Severe mutations include those associated with the neuronopathic forms of the disease. Mild mutations are those that are always associated with type 1 disease, even when they are present in combination with a severe or a lethal mutation.

Severe mutations tend to produce enzymes with decreased stability and severely reduced catalytic activity, while mild mutations (e.g., N370S) lead to mutant GBA with reduced activity but nearly normal stability.

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It is difficult to classify a mutation as mild because most type 1 GD patients bear the common N370S mutation. As the presence of this mutant allele implies by itself a type 1 phenotype, the severity of the accompanying mutation is difficult to ascertain. Moreover, most reported GD genotypes are heterozygous compounds, and so it is difficult to correlate the clinical findings with either of the 2 mutations, particularly in the absence of expression experiments.

Here we report on 3 patients of Italian origin affected with type 1 GD. Two of them were homozygous for 2 different, previously undescribed mutations: I402T and V375L, and the third one was heterozygous for the I402T mutation. The homozygosity for I402T prompted us to compare some properties of the mutant enzyme in cells from this patient to those from patients homozygous for N370S. The lack of available material from the patient homozygous for V375L precluded further enzymatic characterization.

PATIENTS

Patient 1

This patient was from Catania (Sicily, Italy) and his parents were consanguineous. An older sister had hepatosplenomegaly and died at the age of 64 without a specific clinical diagnosis. The patient is now 45 years old and he presented the first symptoms at age 37 years when he complained of asthenia and fatigue. Physical examination at 41 years showed a marked visceral enlargement with liver palpable at 7 cm and spleen at 10 cm below the costal margin. Blood cell counts showed thrombocytopenia (32,000 platelets per microliter). Serum level of acid phosphatase was high (15.2 U/l with normal values < 4.7 U/l). A bone marrow biopsy showed typical Gaucher cells. The patient has no skeletal involvement. He was treated with enzyme replacement therapy, and a gradual improvement of the hematological parameters was observed (platelet count: 105,000 per microliter). Progressive reduction of the visceral enlargement was demonstrated both on clinical and ultrasound examination.

Patient 2

This patient was a 36-year-old man from the same region as patient 1. His parents were not consanguineous. Clinical symptoms began at the age of 33 years with recurrent epistaxis and general weakness. Clinical evaluation at 36 years demonstrated moderate visceral enlargement (liver and spleen 4 cm below the costal margin) and reduced platelet counts (90,000 per microliter). Gaucher cells were detected in a bone marrow biopsy. Skeletal radiographs were normal.

Patient 3

Patient 3 is now 44 years old. He also had an Italian origin but no family data are available. At the time of diagnosis (39 years), he presented with asthenia, stroke-like episodes with paresia of his right arm, and thrombocytopenia. Physical examination demonstrated spleen and liver enlargement and Gaucher cells were detected in the bone marrow. The patient was splenectomized and the anatomical examination of the spleen confirmed Gaucher disease.

Patients Homozygous for N370S

For comparative enzymatic studies, 6 Spanish type 1 GD patients (N370S/N370S) were analyzed. Four of them have been described previously [I.2, I.7., I.9, and I.12 in Cormand et al., 1995].

MATERIALS AND METHODS

Skin fibroblast cultures were established according to routine procedures in Eagle's minimum essential medium. Leukocytes were prepared according to the method of Skoog and Beck [1956]. Sphingolipid activator protein C (Saposin C, Gaucher activator) was partially purified from a human brain [Chabás et al., 1987].

Biochemical Analyses

Enzymatic activity in fibroblasts and leukocytes was determined in citrate buffer (0.04 M, pH 5.5) using 4MU-β-glucopyranoside as the substrate (MU-Glc, 3 mM) in the presence of sodium taurocholate (T, 0.3% w/v) and Triton X-100 (TX, 0.24% w/v) in a reaction mixture of 0.15 ml.

Km studies. The reaction mixture contained fibroblast extract in a constant amount of enzyme activity and MU-Glc at a concentration range of 0.4–10 mM in citrate buffer (0.04 M, pH 5.5). Km values were determined from Lineweaver-Burk plots.

Heat stability. The fibroblast extracts (20–30 μg protein) in 0.5 M citrate buffer, pH 5.5, were placed at 50°C and enzyme activity was assayed in samples withdrawn at 0, 5, 10, 20, 30, and 60 minutes as described above.

Effect of natural activator. Stimulation of fibroblast glucocerebrosidase by the combination of Saposin C (SAP, 150 μg) and phosphatidylserine (PS, 5 μg), or by PS alone, was carried out using MU-Glc (2.5 mM) in citrate buffer (0.025 M, pH 5.0) in a final volume of 0.2 ml.

Other enzyme assays. Plasma chitotriosidase activity was examined [Hollak et al., 1994]. Activity of β-xylosidase in fibroblasts (50–75 μg protein) was measured with 4MU-β-xylopyranoside (1.6 mM in 0.04 M citrate buffer, pH 5.0) in the presence of T and TX in a final volume of 0.13 ml.

Molecular Analyses

High molecular weight DNA was prepared from peripheral blood leukocytes using the salting out procedure of Miller et al. [1988]. The A6144G polymorphism, in intron 9 of the GBA gene was studied by *Hha*I digestion of a polymerase chain reaction (PCR)-amplified product. Gene-specific primers (sense: nt 5904–5923, antisense: 6655–6690) were used to amplify a 787 bp

fragment [Sidransky et al., 1992]. The alleles were designated as "+" (*Hha*I site present) or "–" (*Hha*I site absent) according to Beutler et al. [1992].

The common N370S mutation was detected by "mismatched PCR" using a 5′ primer mismatched at one nucleotide in order to create a *Xho*I restriction site when the mutation was present [Beutler et al., 1990]. Digested PCR products were subjected to electrophoresis on a 4% NuSieve GTG agarose gel (FMC, Rockland, ME).

Mutation L444P was detected by PCR amplification followed by *Nci*I digestion of the product and electrophoresis on a 1.2% agarose gel [Sidransky et al., 1992]. Mutation D409H was detected by allele-specific oligonucleotide (ASO) hybridization as described by Cormand et al. [1995].

PCR amplification and SSCP analysis of 14 DNA fragments covering all 11 exons of the GBA gene and their flanking sequences were performed on genomic DNA from the patients and relatives. All primer pairs were chosen to amplify the GBA gene and not the highly homologous pseudogene. The size of the amplified fragments ranged from 139 to 292 bp. For all fragments, the PCR reaction was performed in a volume of 50 μl containing 100 ng of genomic DNA, 1 U of Dynazyme DNA polymerase (Finnzymes Oy, Finland). 200 μM dNTPs, and 20 pmol of each primer, in the buffer recommended by the manufacturer. The PCR program consisted of 35 cycles of denaturation at 94°C for 40 seconds and a single annealing/extension, step at 55°C for 30 seconds.

For the single-strand conformation polymorphism (SSCP) analysis, 1 μl of the PCR product was mixed with 6 μl of 95% formamide, 0.05% xylene cyanol, 0.05% bromophenol blue, 20 mM EDTA solution. The samples were then denatured by incubation at 80°C for 3 minutes and placed on ice. Electrophoresis was carried out using a 18 × 24 cm nondenaturing polyacrylamide gel. Four SSCP conditions were tested in each fragment, combining different polyacrylamide concentrations (8 or 12% acrylamide:bisacrylamide 29:1), different glycerol concentrations in the gel (0% or 5% glycerol), and 2 running conditions (RT at 200 V, or 4°C at 300 V, always 12 hours). Single and double DNA strands were revealed by silver staining as follows: the gel was incubated for 5 minutes with a 10% ethanol solution and for 3 minutes with a 1% HNO_3 solution. After washing in deionized water, it was incubated for 20 minutes with a 12 mM $AgNO_3$ solution. The gel was washed again and incubated (up to 10 minutes) with a freshly prepared mixture of 280 mM Na_2CO_3 and 0.02% formaldehyde. The gel was finally soaked in 10% acetic acid solution and dried on Whatman 3MM paper.

Fragments that showed aberrant pattern compared to normal in the SSCP test were amplified, purified (Wizard PCR Preps, Promega, Madison, WI), and directly sequenced by fluorescent dideoxy cycle sequencing (ABI 373A Fluorescent DNA Sequencer, Perkin Elmer Cetus, Norwalk, CT). Genomic sequence numbering is according to Horowitz et al. [1989]. cDNA numbers start from the first ATG.

Computational Analysis

The secondary structure for the normal and mutant proteins was predicted by the method of Chou and Fasman [1978] using the GCG package [Devereux et al., 1984].

RESULTS

Biochemical Analysis

In the presence of T and TX, high residual β-glucosidase activity was detected in leukocytes and in cultured fibroblasts from patient 1, close to the lower limit presented by healthy carriers for GD and higher than in type 1 patients bearing the mild mutation N370S at homozygosity. Patient 2 fibroblasts showed an intermediate level of residual enzymatic activity while in patient 3 leukocyte activity was that expected for the disease (Table I).

We analyzed some properties of the fibroblast enzyme and compared them to those in 4 patients bearing genotype N370S/N370S as well as in control cells (Table II). In the presence of the natural activators PS and SAP, β-glucosidase in patient 1 fibroblasts was stimulated about 2-fold (as compared to enzyme activity in the absence of these activators), similarly to the enzyme from control cells. Under these experimental conditions, enzyme activity from patients with the N370S/N370S genotype increased 2.5 to 3.7-fold (mean: 2.9-fold). PS alone also stimulated β-glucosidase activity in cells from patient 1 and from the N370S homozygous patients to the same extent (about 1.6-fold, data not shown).

The apparent Km value of β-glucosidase for MU-Glc was almost identical in patient 1, the N370S homozygous patients, and controls. Analyses of heat stability of β-glucosidase showed that enzymes from patient 1, the N370S homozygous patients, and controls are similarly stable for 1 hour at 50°C (92–100% of initial activity). Estimations of some secondary biochemical abnormalities such as the plasma chitotriosidase activity in patient 1 demonstrated a 166-fold increase (control mean: 79 nmol/h/ml). Fibroblasts from this patient also showed a high residual β-xylosidase activity (38% of mean control) while in the N370S/N370S patients this activity was only 4% of mean value (control mean: 7.5 nmol/h/mg protein) (data not shown).

TABLE I. Residual β-Glucosidase Activity in Patients Bearing the Novel Mutations I402T and V375L and in Patients Homozygous for N370S

	Genotype	Activity (nmol/h per mg protein)[a]	
		Leukocytes	Fibroblasts
Patient 1	I402T/I402T	4.0	94
Patient 2	N370S/I402T		65
Patient 3	V375L/V375L	1.8	
Type 1 GD[b]	N370S/N370S	1.7 ± 0.2 (6)	23 ± 6 (4)
Controls[b]		6.2 ± 1.3 (42)	261 ± 102 (56)

[a]Enzyme activity measured in the presence of T and TX.
[b]Enzyme activity mean ± SD. Number of cases in parentheses.

TABLE II. Comparative Enzymatic Properties of β-Glucosidase Activity in Fibroblasts From Patient 1 and From Patients Homozygous for the N370S Mutation

	Genotype	Activity (nmol/h/mg)[a]	Activity ratio (-fold)[b]	Km 4MU-Glc (mM)	Heat stability (%)[c]
Patient 1	I402T/I402T	107	2	4.1	92
Type 1 GD (4)	N370S/N370S	103 ± 44	2.5–3.7	3.8 ± 1.7	100
Controls		343 ± 122	2	3.9 ± 0.3	91–100

[a]Activity assayed in the presence of PS and saposin C (SAP). Activity expressed as mean ± SD.
[b]Ratio between the activity assayed in the presence of PS and saposin C (SAP) and in the absence of both activators.
[c]% initial activity after 1 hour at 50°C.

Mutation Analysis

An initial screening for the previously described N370S, L444P, and D409H mutations in the 3 Italian GD patients showed that patient 2 was heterozygous for the common N370S amino acid substitution. None of these mutations was detected in the other 2 individuals.

In order to identify the other mutations, the 11 exons of the GBA gene were screened by SSCP analysis. The PCR products included all the coding region of the gene, the corresponding exon/intron boundaries, part of the promoter, and the upstream polyadenylation signal. Abnormal SSCP patterns were observed only in exon 9 in all 3 patients and in some relatives (Figs. 1a, 2a).

Sequence analysis demonstrated that patient 1 was homozygous for a previously undescribed missense mutation due to a T - >C transition (Fig. 1b) at genomic nucleotide 5937 (cDNA 1322). This mutation leads to an isoleucine to threonine substitution at residue 402 in the mature enzyme. An unaffected brother was found to be a carrier of this mutation.

Patient 2 was a compound heterozygote for the same amino acid substitution (Fig. 1b) together with the N370S mutation. Three unaffected brothers were genotyped as I402T/+, N370S/+, and +/+. Besides, a daugh-

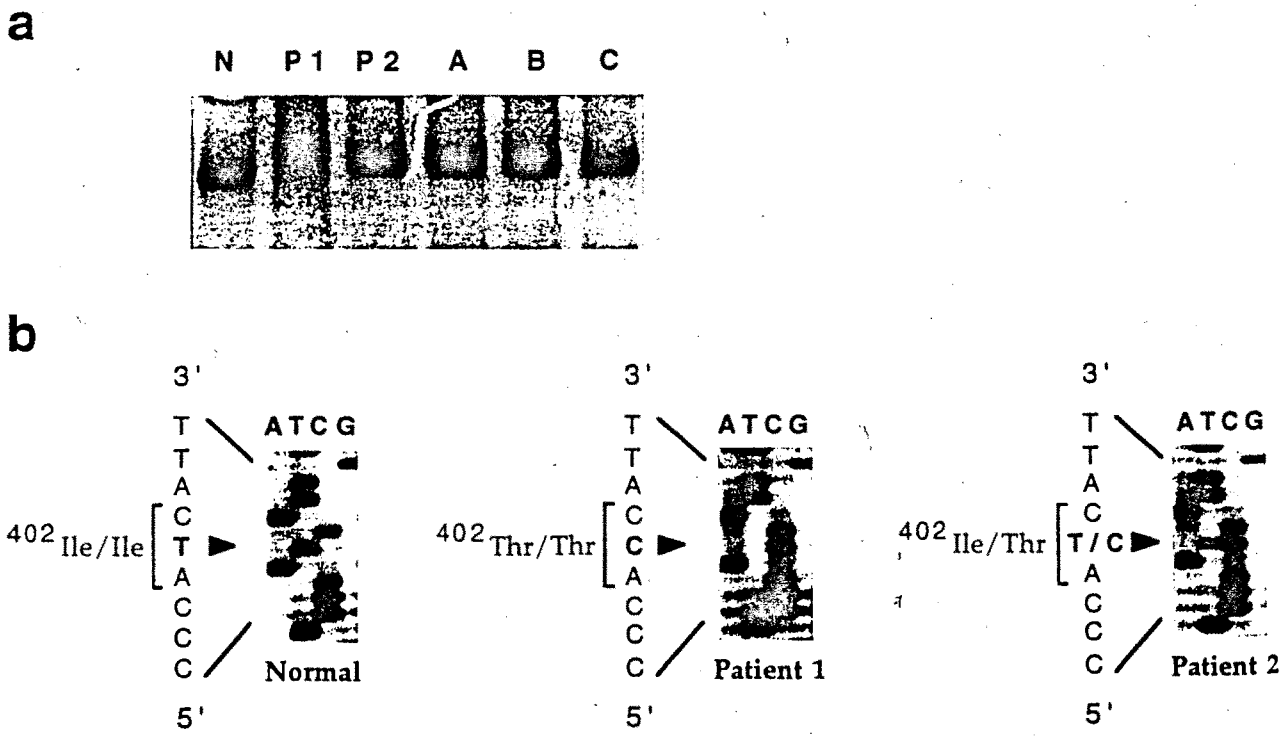

Fig. 1. Identification of mutation I402T by SSCP analysis and sequencing. **a:** SSCP analysis of PCR-amplified DNA fragments containing exon 9 of the GBA gene. Primer sequences (5' - >3'): sense: ACTG-GAACCTTGCCCTGAAC (nt: 5871–5890); antisense: ATAGGCCTGGTATGGAATGG (6025–6044). The samples were run in a nondenaturing 12% polyacrylamide gel for 12 hours at 300 V, 4°C. Only the single strands are shown. The bands on the gel were revealed by silver staining. **Lane N:** normal control; **lanes P1, P2:** patients 1 and 2, respectively; **lane A:** brother of patient 1; **lanes B, C:** brothers of patient 2. **b:** Direct sequence of exon 9 PCR products from patients 1 and 2 and from a control individual. Patient 1 (central panel) is homozygous for a T-to-C transition in the second position of codon 402, leading to a Thr for Ile amino acid substitution. Patient 2 (right panel) is heterozygous for the same mutation.

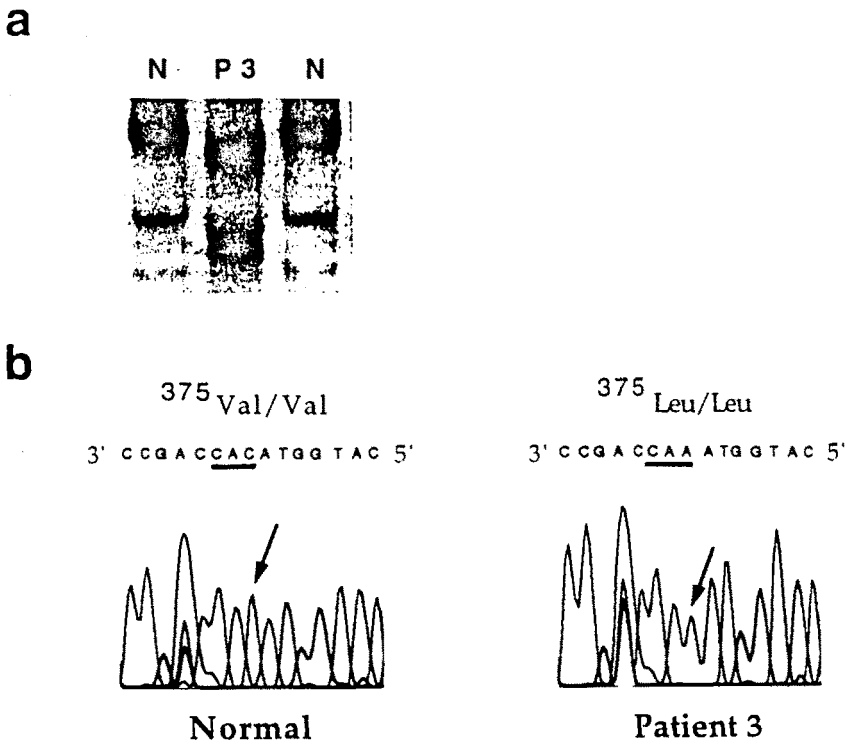

Fig. 2. Identification of mutation V375L by SSCP analysis and sequencing. **a:** SSCP analysis of exon 9 PCR products. Primer sequences (5' – >3'): sense: GTGTTGAGCCTTTGTCTCTT (nt: 5800–5819); antisense: GATGGGACTGTCGACAAAGT (5919–5938). **Lane N:** Normal individuals; **lane P3:** patient 3. The samples were run in a nondenaturing 12% polyacrylamide gel for 12 hours at 300 V, 4°C. **b:** Reverse sequence of exon 9 PCR products in patient 3 and in a control individual. Patient 3 (right panel) is homozygous for a G – >T transversion in the first position of codon 375, resulting in the substitution of leucine for valine.

ter of the patient was a carrier of the I402T mutation, while a son bore mutation N370S (data not shown).

Patient 3 was homozygous for another novel mutation. This is a G – >T transversion (Fig. 2b) at nucleotide 5855 of the gene (nucleotide no. 1240 of the cDNA, starting from the first ATG) substituting leucine (TTG) 375 of the mature protein for valine (GTG).

The predicted secondary structure for the 2 new mutant proteins did not show any change compared to the normal protein. The nucleotide changes leading to mutations I402T and V375L are not present in the pseudogene sequence. As they do not create or abolish any restriction site, the screening of a number of unaffected individuals was performed by SSCP analysis, comparing the patterns obtained with those from the patient samples. This screening failed to detect any of these changes in 70 normal chromosomes.

The analysis of the *Hha*I intragenic polymorphism showed that the 3 I402T alleles found in patients 1 and 2 are associated with the "–" variant, and the 2 V375L alleles of patient 3 are associated with the "+" variant (data not shown).

DISCUSSION

One important aim of the molecular analysis of the GBA gene is to correlate the nature of the mutations with the clinical manifestations of the disease, in anticipation to prognosis and counseling. However, this is difficult in a recessive disorder with a well-known allelic heterogeneity such as GD. Only when the mutation under study is present either at homozygosity or compounded with a null or a severe mutation, can conclusions be clearly drawn.

Homozygous mutations are extremely rare in GD patients with the sole exception of N370S, L444P and, to a lesser extent, D409H. Patients with the N370S/N370S genotype have a very mild form of the disease without neuronopathic involvement. Among the neuronopathic types of the disease, the most frequent homozygote genotype is L444P/L444P. Although this genotype produces neurologic disease in most cases, the phenotypic expression is heterogeneous including both type 2 and 3 patients. At younger ages, it may be associated with aggressive manifestations reported as type 1 GD since neurological signs may appear later [Horowitz and Zimran, 1994a]. Recently, some particular phenotypic manifestations, such as cardiovascular calcifications and supranuclear ophthalmoplegia, have been reported in patients homozygous for D409H [Chabás et al., 1995; Abrahamov et al., 1995; Beutler et al., 1995]. Three reportedly mild mutations, R496C [Kawame et al., 1992]. P122S [Beutler et al., 1993], and N188S [Kim et al., 1996] were found at homozygosity in 3 type 1 patients. Regarding the R496C/R496C case, a Japanese patient, no clinical data were reported except for his assignment to type 1. The P122S/P122S geno-

type produces severe visceral disease, and the lack of neuronopathic involvement could be due to the age of the patient (3 years at diagnosis), making it difficult to classify this allele as mild. The N188S/N188S patient was diagnosed at age 17 years with asymptomatic hepatosplenomegaly and no bone disease. From these data, N188S is the only mutation of these 3 that can be undoubtedly classified as a mild mutation.

To date, about 10 GD mutations in addition to the common N370S have been classified as mild [Balicki and Beutler, 1995], in most cases because they were found in compound heterozygosity with a known severe or lethal mutation in type 1 patients. Nevertheless, most type 1 GD patients have at least one allele with the frequent N370S mutation, in both Ashkenazi Jewish [Horowitz and Zimran, 1994b], and non-Jewish populations [Beutler and Gelbart, 1993]. As the presence of N370S is always associated with nonneuronopathic disease, it is impossible to assess the severity of the second mutation in a compound heterozygote bearing N370S.

Mutations I402T and V375L described here, in addition to N370S and N188S, are the only clearly mild mutations that have been analyzed in a homozygous state. Two facts strongly suggest that the 2 amino acid substitutions identified are indeed disease-causing mutations: 1) the changes were found in GD patients and their family members but not in unrelated healthy individuals and 2) the examination of the entire coding region, part of the putative promoter [Doll et al., 1995], all the splice sites, and the first polyadenylation signal of the gene did not show any other mutations.

Our results indicate a marked similarity in the catalytic properties and heat stability of the I402T and N370S mutant fibroblast enzymes. Stimulation of both mutant enzymes by either the physiological activators PS and SAP (activity ratio in Table II) or PS alone (1.6-fold) is also similar. The 2-fold activation observed with the I402T mutant β-glucosidase (in patient 1) by the combination of PS and SAP would then indicate a rather moderate stimulatory effect of SAP. In this regard, a very poor stimulatory response to SAP with the N370S mutant enzyme had been demonstrated in expression studies of β-glucosidase cDNA encoding this mutation [Ohashi et al., 1991; Grace et al., 1994]. These authors state that the responses to PS and SAP were very similar when the overexpressed mutagenized or the natural mutant fibroblast enzymes for N370S were assayed. These findings would validate the analysis of the natural mutant enzyme when a homozygous patient is identified.

However, some differences between mutations N370S and I402T have been observed. The presence of the I402T allele in cells in patients 1 and 2 results in a higher residual activity when T is used as the enzyme activator.

The presence of mutation I402T in 2 unrelated patients from the same geographic region in Sicily suggests that this GD allele might be quite frequent in this small area. The relative isolation of this population, both by historical and geographical reasons [Guglielmino et al., 1991; Rodriguez-Larralde et al., 1994], could explain the expansion of some mutations due to founder effect and genetic drift. Nevertheless, more cases are needed to support this hypothesis. The analysis of the intragenic *Hha*I polymorphism shows that this mutation is associated with the common "–" variant in patients 1 and 2. In patient 3, mutation V375L is associated with the uncommon "+" variant in both alleles, but no familial information is available. It is tempting to speculate about a common origin for the I402T alleles in the unrelated patients 1 and 2 and for the 2 V375L alleles in patient 3, although further data should be required.

The analysis of additional GBA mutations at homozygosity and the study of their phenotypic effects in the patients may provide more insight into the genotype/phenotype correlation for GD.

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Mutation Analysis of Gaucher Disease Patients From Argentina: High Prevalence of the RecNciI 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, RecNciI, and RecTL. This allowed the identification of 77.4% of the GD alleles: N370S and RecNciI were the most prevalent mutations found (46.8% and 21% respectively). Southern analysis demonstrated three distinct patterns for the RecNciI 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 RecNciI 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; RecNciI 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 $j = \Sigma x_i^2$, 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

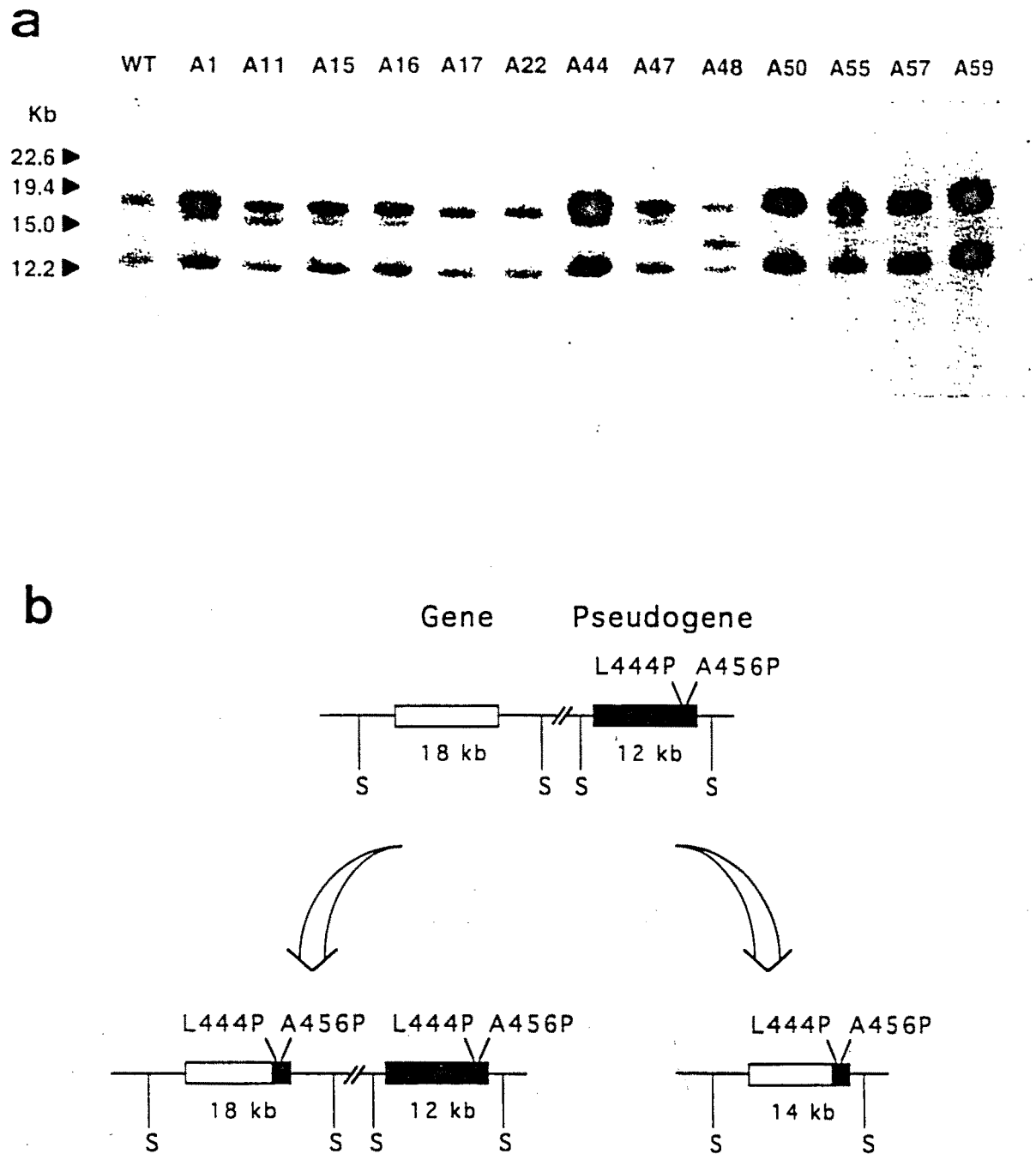

Fig. 1. a: Southern blot analysis of genomic DNA samples digested with *Ssp*I. The lane on the left is a normal control with two bands of ~18 and 12 kb. Patients A17, A22, A50, A57, and A59 have the normal two-banded pattern. Patients A1, A11, A15, A16, A44 (and his brother A47) and A55 show an extra 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

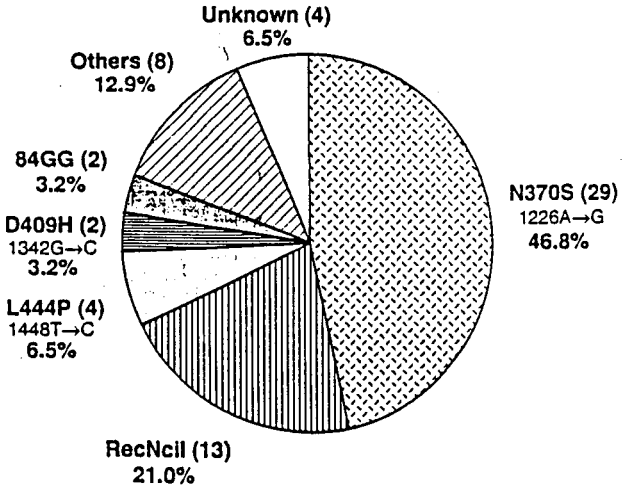

Fig. 2. Prevalence of mutations among 31 unrelated GD patients mainly from Argentina. The numbers in brackets represent number of GD alleles.

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	-*MspI*/-*Nci*I	Chabás et al., 1996
I161S	**T → G**	**3393**	**599**	**6**		**First report**
G202R	G → A[c]	3515	721	6	-*MspI*/-*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.

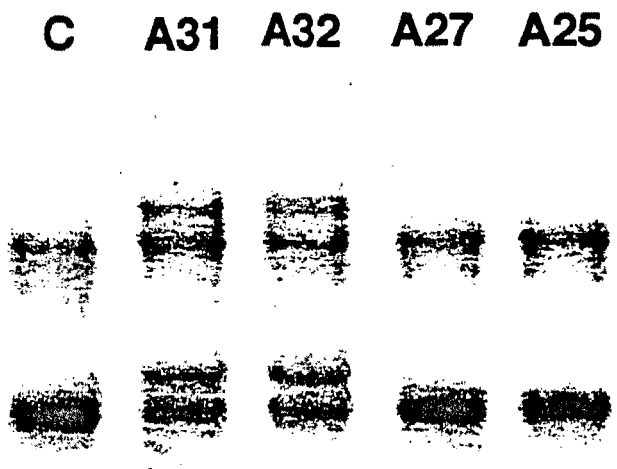

Fig. 3. Detection of the new mutation G265D (911G→A) by SSCP analysis. The PCR-amplified fragment corresponds to exon 7b (3' region of exon 7). The genotype for the sibs A31 and A32 is N370S/G265D, while those of patients A27 and A25 are N370S/I161S and N370S/D399N, respectively. Mutation I161S is in 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	Σx_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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Recurrence of the D409H mutation in Spanish Gaucher disease patients: description of a new homozygous patient and haplotype analysis

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Abstract

Gaucher disease results, in most patients, from mutations in the gene encoding glucocerebrosidase. Mutation D409H is the third most frequent in Spanish patients, accounting for 5.7% of all mutated alleles. This allele is associated mainly with the neurological forms of the disease. Recently, homozygosity for the D409H mutation has been associated with a particular phenotype, including specific cardiovascular symptoms. Here we report a second Spanish patient bearing the D409H/D409H genotype with a very early manifestation of the disease. The patient started enzyme replacement therapy at 3 months of age. A common origin for the Spanish D409H alleles was ruled out by haplotype analysis using an internal polymorphism of the glucocerebrosidase gene and two external microsatellite markers.

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Keywords: Gaucher disease; mutation D409H; haplotype analysis; enzyme replacement therapy

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Gaucher disease (GD) is a clinically heterogeneous sphingolipidosis caused mainly by mutations in the gene encoding lysosomal glucocerebrosidase (GBA). Mutations in the GBA gene may be classified as lethal, severe, or mild on the basis of the severity of their phenotypic effect.[1] A genotype-phenotype correlation has been established for the most common mutations, such as N370S (1226A→G) with a non-neuronopathic phenotype or L444P (1448T→C) with a severe phenotype.[2] Recently, homozygosity for the D409H mutation (1342G→C) has been associated with a unique form of type 3 Gaucher disease in patients with origins as diverse as Spanish,[3] Arab,[4] British/German,[5] and Japanese.[6] They all had oculomotor apraxia and a devastating valvular heart disease. Common manifestations of GD were not evident and minimal visceral organomegaly, or even absence, was reported in most patients. Corneal opacity was also mentioned in the Spanish, Arab, and Japanese patients while some other features, such as sensorineural deafness, hernia, and deformed toes, found in patients with mucopolysaccharidosis or glycoprotein disorders, were only observed in the Japanese cases.

In total, only six unrelated patients with the confirmed D409H/D409H genotype have been described.[1-6] We now report a second Spanish patient homozygous for the D409H mutation with a very early manifestation of GD, presenting exclusively with massive hepatosplenomegaly. D409H is the third most common mutation in the Spanish GD population[7] and we have identified six unrelated families bearing this mutation. Although a common origin for all D409H alleles was ruled out because of the association with both the "+" and "−" internal haplotypes,[3 8] we carried out haplotype analyses using highly informative flanking markers[9] to evaluate the degree of recurrence of this mutation.

Case report

The patient, now 13 months old, was admitted to hospital at the age of 6 days because of respiratory distress. Hepatosplenomegaly was then detected (spleen 3-3.5 cm and liver 1.5-2 cm below the costal margin). *X* rays of the chest, bone metaphyses, and skull were normal. Multiple haemorrhages in the retina were observed. The patient was readmitted at the age of 1.5 months because of massive hepatosplenomegaly (spleen 7 cm and liver 5 cm below the costal margin) with abdominal colic pains. Serology was negative. Blood counts (haemoglobin, haematocrit, red blood cells) were just below normal values. Chitotriosidase activity was raised (1467 nmol/h × ml, controls 31.6 (SD17.2)). The blood clotting parameters were normal as well as a bone marrow aspirate. Neurological evaluation (electroencephalography, brain auditory evoked response, and CT of the brain) was normal.

Acid β-glucosidase activity was decreased to 23% (leucocytes) and 3.8% (fibroblasts) of controls. Urine samples were not available.

The patient started enzyme replacement therapy with Alglucerase (Ceredase®) at 3 months of age. At the age of 13 months neurological examination and ocular motility were normal. Echocardiography was also normal. Hepatomegaly was drastically reduced and the spleen was 3 cm below the costal margin.

Mutation analysis

Genomic DNA amplification by PCR and SSCP analyses were performed as previously described.[10] The presence of an altered pattern similar to that found in our previous case[3] and subsequent confirmation by sequencing showed that the patient is homozygous for the D409H mutation. Both parents are heterozygous for this mutation. The parents are not consanguineous and are from a different region

of Spain from the previously reported family. A prenatal diagnosis was recently requested by this family. Deficiency of acid β-glucosidase activity and homozygosity for mutation D409H suggested an affected fetus. The pregnancy was terminated and the diagnosis of Gaucher disease was confirmed in the fetal tissue.

Haplotype analysis on chromosomes bearing the D409H mutation

Haplotype analysis was carried out on eight alleles bearing D409H, including the case reported here (patient 1), a previously undescribed patient (patient 6), and four other patients reported elsewhere[3 8] (table 1). The 6144A→G polymorphism in intron 9 of the GBA gene was analysed by *Hha*I digestion of a PCR amplified product. Gene specific primers (sense: nt 5904-5923, antisense: 6655-6690, sequence according to Horowitz *et al*,[11] EMBL/GenBank accession number J03059) were used to amplify a 787 bp fragment.[12] The alleles were designated "+" (*Hha*I site present) or "−" (*Hha*I site absent), following Beutler *et al.*[13]

The association of D409H with either the "+" or the "−" variants of the 6144A→G polymorphism was studied. Phase was established either by genotyping the parents or by cloning a DNA fragment containing both the mutation and the polymorphic site. Results of the analysis of the *Hha*I polymorphism for some of these patients have been previously presented.[8] Only one of the alleles was associated with the "+" allele while seven were associated with the "−" allele of the internal polymorphism (table 1).

Two external microsatellite markers, D1S2140 and D1S2624, known to be at 0 and 1.6 cM respectively from the GBA gene[9] were used to genotype the eight D409H chromosomes. These markers belong to the MapPairs set (Research Genetics) and were analysed according to the manufacturer's recommendations. Marker D1S2140 has seven alleles with the following sizes: allele 1, 232 bp; 2, 244; 3, 248; 4, 252; 5, 254; 6, 256; and 7, 260. Marker D1S2624 has five alleles: allele 1, 209 bp; 2, 205; 3, 211; 4, 203; and 5, 207. The eight D409H chromosomes presented six different haplotypes (table 1). Only patients 1 and 5 share the same one. In addition, patient 2 is homozygous for all three loci. Linkage disequilibrium between D409H and each of the markers was not significant (data not shown).

Table 1 Haplotype analysis for the 6144A→G polymorphism and markers D1S2140 and D1S2624 in patients bearing the D409H mutation

Patients	*GBA mutations*	*6144A→G*	*D1S2140*	*D1S2624*
1 (case report)*	**D409H**	**−**	**4**	**1**
	D409H	**−**	**1**	**4**
2 (type 3)†	**D409H**	**−**	**4**	**4**
	D409H	**−**	**4**	**4**
3 (type 1)‡	**D409H**	**−**	**7**	**1**
	E326K	**+**	**4**	**1**
4 (type 2)‡	**D409H**	**+**	**4**	**3**
	R120W	**+**	**6**	**3**
5 (type 3)‡	**D409H**	**−**	**1**	**4**
	L444P	**+**	**3**	**4**
6 (type 1)*	**D409H**	**−**	**1**	**2**
	G377S	**−**	**4**	**1**

*This paper. †Reference 3. ‡Reference 8. Haplotypes for D409H chromosomes are shown in bold.

Discussion

A very limited number of patients homozygous for mutation D409H have been reported so far. The patient described here was diagnosed at 1.5 months of age because of massive hepatosplenomegaly. Despite the early presentation of the disease, the absence of neurological signs ruled out the acute, infantile form of GD. Characteristic findings in patients bearing the D409H/D409H genotype, such as ophthalmoplegia and cardiovascular abnormalities, were not noticed in our patient, probably because of the young age. Ophthalmoplegia had been noticed in early childhood in some patients homozygous for mutation D409H[4 6] while in others gaze palsy started at 15-16 years of age.[3] In these patients, cardiac involvement consisting of valvular calcifications was also apparent in late childhood.[3 4]

The patients of Japanese origin[6] provide the most atypical clinical picture as they present a combination of clinical signs which appear just as isolated features in some patients of other origins. The massive visceromegaly of the patient reported here, absent in the Japanese patients and moderate in other cases, increases the range of heterogeneity in patients with the D409H/D409H genotype.

To date, there have been no published data on a patient with this genotype receiving enzyme replacement therapy. The case reported, now at the age of 13 months and after 10 months of treatment, shows no signs of cardiac involvement. As expected, the splenomegaly and hepatomegaly have been reduced and the patient shows a normal haemogram. If the patient does not present cardiac disease later in life, as did all the other D409H/D409H patients previously reported, it will be difficult to assess if this is because of the early treatment or phenotypic heterogeneity. It should be noted that reversible cardiomyopathy with enzyme replacement therapy has recently been reported in a 5 year old patient with type 1 GD.[14]

For haplotype analysis, five other patients were analysed, including one previously undescribed (patient 6). This patient is a 47 year old type 1 woman who bears genotype D409H/G377S (1246G→A). This is the first description of mutation G377S in the Spanish population. Interestingly, it is the third most prevalent mutation in Portugal.[15]

In order to determine whether mutation D409H was present by descent from a single origin or the result of independent mutational events, we carried out haplotype analysis using one of the internal polymorphisms of the GBA gene and two external but very close markers on the eight alleles found in the Spanish population. The analysis of the internal polymorphism showed that only one D409H allele was associated with the "+" allele while the rest were associated with the "−"allele, as previously determined,[8] with some of the samples. This ruled out a single origin for the Spanish D409H mutation, but a founder effect for the seven chromosomes bearing the "−" allele of the *Hha*I internal polymorphism could not be excluded. However, the analysis of the highly polymorphic markers D1S2140 and D1S2624,

known to be at 0 and 1.6 cM respectively from the GBA gene,[9] showed that the D409H mutations are associated with different haplotypes. Only haplotype D409H-1-4 appeared in two different patients (table 1). The two chromosomes of patient 2 showed the same haplotype, suggesting consanguinity, although the family was not aware of this. The lack of association between D409H and the markers indicate that D409H is a recurrent mutation. As the site of the mutation is not a CpG dinucleotide, known to be a mutational hot spot, and as it is present in the pseudogene, gene conversion events could explain this recurrence.

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HUMAN MUTATION 11:295–305 (1998)

RESEARCH ARTICLE

Molecular Analysis and Clinical Findings in the Spanish Gaucher Disease Population: Putative Haplotype of the N370S Ancestral Chromosome

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Gaucher disease (GD) is an autosomal recessive disorder caused by mutations in the lysosomal β-glucocerebrosidase (GBA) gene. As the disease is particularly prevalent among Ashkenazi Jews, most studies have been carried out on this ethnic group. In the current study, we present a mutation analysis of the GBA gene in Spanish patients together with the clinical findings. We conducted a systematic analysis in 53 unrelated GD patients. The GBA gene was initially scanned for nine previously described mutations by ASO hybridization or restriction analysis after PCR amplification. The remaining unidentified alleles were screened by nonisotopic PCR-SSCP analysis and sequenced. This approach allowed the identification of 101 of 106 GD alleles (95.3%) involving 24 different mutations, 11 of which are described for the first time: G113E (455G→A), T134P (517A→C), G389E (1283G→A), P391L (1289C→T), N392I (1292A→T), Y412H (1351T→G), W(-4)X (108G→A), Q169X (662C→T), R257X (886C→T), 500insT, and IVS5+1G→T. Most mutations are present in one or few GD chromosomes. However, two mutations, N370S (1226A→G) and L444P (1448T→C), are very frequent and account for 66.1% of the total number of alleles. Linkage disequilibrium was detected between these two mutations and an intragenic polymorphism, indicating that expansion of founder alleles occurred in both cases. Analysis of several microsatellite markers close to the GBA gene allowed us to establish the putative haplotype of the ancestral N370S chromosome. Hum Mutat 11:295–305, 1998. © 1998 Wiley-Liss, Inc.

KEY WORDS: Gaucher disease; GBA gene; mutation screening; linkage disequilibrium; Spanish patients; N370S mutation; ancestral haplotype

INTRODUCTION

Gaucher disease (GD) is a lysosomal glycolipid storage disorder characterized by the accumulation of glucosylceramide in phagocytes due to deficient activity of β-glucocerebrosidase (E.C. 3.2.1.45). It is inherited as an autosomal recessive trait. Three major types of Gaucher disease have been described on the basis of the absence (type I) or presence and severity (types II and III) of primary central nervous system involvement. The disease is particularly frequent in the Ashkenazi Jewish population in which the incidence of GD is estimated as 1 in 850 (Beutler and Grabowski, 1995), while in other populations the frequency ranges between 1 in 40,000 and 1 in 60,000 (Grabowski, 1993).

In most of the cases described so far, GD is caused by mutations in the GBA gene encoding β-glucocerebrosidase. More than 70 mutations have been described in the GBA gene to date (Beutler and Gelbart, 1997). Mutation N370S (1226A→G) is the most prevalent among Ashkenazi Jews (approximately 70% of GD alleles) (Beutler et al., 1992a; Horowitz et al., 1993; Sibille et al., 1993) and is also frequent in other populations (22–54%) (Walley et al., 1993; Cormand et al., 1995; Michelakakis et al., 1995; Amaral et al., 1996; Tylki-Szymanska et al., 1996; le Coutre et al., 1997).

Twelve polymorphic sites have been detected

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within the *GBA* gene (Beutler et al., 1992b). All are in linkage disequilibrium and constitute only two major haplotypes. N370S has always been found associated with the – haplotype (Zimran et al., 1990a), suggesting a single origin for this mutation. The *GBA* gene was recently mapped to a 3.2-cM interval between markers D1S305 and D1S2624 (Cormand et al., 1997b), with no recombination detected between the gene and markers D1S2777, D1S2721, and D1S2140.

We previously analyzed several known GD mutations in 35 Spanish patients (Cormand et al., 1995). In this paper, we present the molecular analysis of the *GBA* gene in 53 patients. This approach allowed the identification of 95% of the mutant alleles, resulting in the detection of 24 different mutations, 11 described for the first time. Clinical findings were also studied to explore the genotype–phenotype correlations. In addition, linkage disequilibrium between the most common mutations and an intragenic polymorphic site was evaluated and flanking STR markers were used to establish a putative ancestral haplotype, in which the common N370S mutation could have first occurred.

MATERIALS AND METHODS

Patients

Fifty-three unrelated Spanish GD patients with different clinical subtypes (42 type I, 8 type II, and 3 type III) were studied. They originate from different locations in continental Spain and the Canary islands. A clinical evaluation was provided by the patient's physician, in most cases including physical examination, routine hematological and laboratory tests, and radiological and neurological studies. In all cases, the GD diagnosis was confirmed by demonstration of low β-glucocerebrosidase activity in leukocytes or cultured fibroblasts. Some of these patients are described elsewhere (Chabás et al., 1995, 1996; Cormand et al., 1995, 1996). Several Argentinian patients (submitted) were included for the analysis of haplotypes associated with mutation N370S. Appropriate informed consent was obtained from all patients.

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 (Cormand et al., 1995).

DNA Isolation

Genomic DNA was prepared from harvested skin fibroblasts, peripheral blood leukocytes, or spleen, using a standard method (Miller et al., 1988).

Detection of Nine Previously Described *GBA* Mutations

All patients were screened for eight known mutations in the *GBA* gene (N370S, L444P [1448T→C], D409H [1342G→C], R463C [1504C→T)], 84GG, IVS2+1, RecTL and Rec*Nci*I). The genomic samples were amplified by polymerase chain reaction (PCR) and examined by either allele-specific oligonucleotide (ASO) hybridization or restriction analysis (Cormand et al., 1995). The gene/pseudogene recombinant alleles were analyzed by determining the presence of the point mutations L444P (1448T→C), A456P (1483G→C) and V460V (1497G→C) for Rec*Nci*I, and D409H (1342G→C), L444P (1448T→C), A456P (1483G→C), and V460V (1497G→C) for RecTL. In addition, the 1263del55 mutation was screened by gene-specific PCR amplification of a 476-bp fragment [sense primer, nt 5569–5588; antisense primer, nt 6025–6044; sequence numbering according to Horowitz et al. (1989)]; and separation of the products on a 2% agarose gel.

PCR Amplification and SSCP Analysis

PCR and single-strand conformation polymorphism (SSCP) analysis of 14 DNA fragments covering the 11 exons of the *GBA* gene, the flanking intronic junctions, part of the promoter region, including the TATA box, and the first polyadenylation signal, was performed from genomic DNA of the patients. The size of the PCR fragments used ranged from 139 bp to 292 bp, 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 *GBA* pseudogene, except in exon 11, for which this was not possible. For gene-specific amplification of this last exon, a nested PCR was performed from a larger, gene-specific PCR fragment. Information on primers is available from the authors.

Although many primers presented several mismatches with the pseudogene sequence, in six of the 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. In these cases, the G/T heteroduplex was avoided, as it is relatively stable (Kwok et al., 1994). Nevertheless, 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 amplifed.

For all fragments, the PCR reaction was performed under the following conditions: 100 ng of template

DNA, 1 U of Dynazyme DNA polymerase (Finnzymes Oy, Finland), 200 μM dNTPs, 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 single annealing/extension step at 55°C for 30 sec.

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

Samples showing abnormal SSCP patterns were reamplified from genomic DNA and the new PCR reactions were subsequently purified by Wizard™ PCR Preps (Promega, Madison, WI) and cloned into pUC18 vector using the SureClone™ Ligation Kit (Pharmacia Biotech, Gaithersburg, MO). In each case, 6–10 clones were sequenced by fluorescent dideoxy cycle sequencing (ABI 373A Fluorescent DNA Sequencer, Perkin Elmer, Norwalk, CT). Alternatively, in four cases, to analyse each allele independently avoiding the time-consuming subcloning steps, one of the abnormal SSCP bands was cut out of the gel after silver staining and eluted in 50 μl of water for 2 hr at 50°C; 20 μl was used in a 100-μl PCR reaction. The PCR fragments were column-purified and sequenced.

Mutations P391L (–*Ava*II), G389E (+*Mbo*II), R359Q (–*Taq*I), Y313H (–*Kpn*I), G202R (–*Msp*I), G195E (+*Hinf*I), R120W (–*Msp*I), and 1451delAC (–*Hga*I) were confirmed by digestion of the corresponding PCR products with the indicated enzyme. The remaining rare GD alleles were confirmed by direct sequencing of PCR-amplified fragments.

Protein Secondary Structure

The secondary structure for the normal and mutant proteins were predicted by the method of Chou and Fasman (1978), using the GCG package (Devereux et al., 1984).

Analysis of the 6144A→G GBA Polymorphism

The 6144A→G polymorphism in intron 9 of the *GBA* gene was analyzed by *Hha*I digestion of a PCR-amplified product. Gene-specific primers (sense: nt 5904–5923, antisense: 6655–6690) were used to amplify a 787-bp fragment (Sidransky et al., 1992). The alleles were designated + (*Hha*I site present) or – (*Hha*I site absent), according to the method of Beutler et al. (1992).

The association of the N370S and L444P alleles with either the + or the – variants of the intragenic 6144A→G polymorphism was studied. In double heterozygous patients, phase was established either by genotyping the parents or by cloning a DNA fragment containing both the mutation and the polymorphic site.

Haplotype Analysis

Several microsatellite markers (D1S2140, D1S2777, D1S2721, D1S2624, and D1S305) (Fig. 1) located close to the *GBA* gene (Cormand et al., 1997b) were used to genotype 24 N370S chromosomes. These markers belong to the MapPairs set (Research Genetics, Huntsville, AL) and were analyzed according to the manufacturer's recommendations.

Statistical Analyses

Allelic associations between the diallelic 6144A→G polymorphism and the common N370S and L444P mutations were evaluated using the $D' = D/D_{max}$ standardized linkage disequilibrium coef-

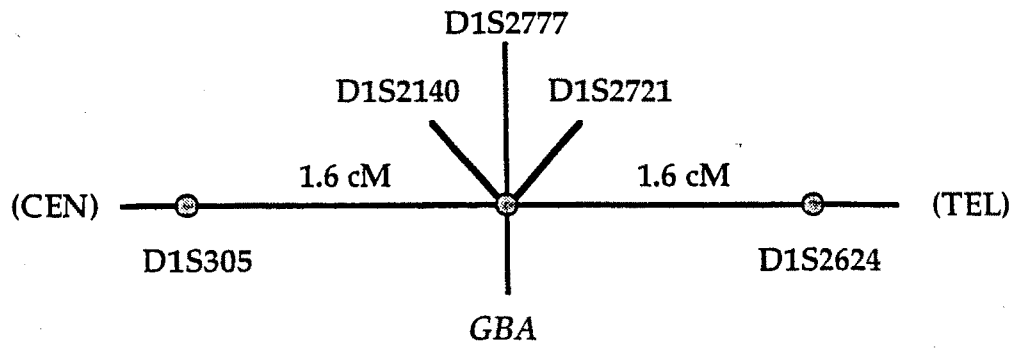

FIGURE 1. Genetic map of microsatellite markers used for linkage disequilibrium and haplotype analysis at 1q21. The genomic region spanned by D1S305 at the centromeric boundary and D1S2624 at the telomeric boundary with the corresponding genetic distances (Dib et al., 1996) is shown schematically. The glucocerebrosidase locus (*GBA*) was recently mapped by the authors close to markers D1S2140, D1S2777, and D1S2721 (Cormand et al., 1997b).

ficient (Lewontin, 1988). The significance of association was tested by a one-sided χ^2 test.

For markers with multiple alleles, saturated hierarchical log-linear models were fitted to the data (Fañanás et al., 1992; E. Monrós and J. Bertranpetit, unpublished data). The λ interaction parameter is an estimate of the degree of association between each allele of a marker and the presence or absence of the disease mutation. The significance of this association was measured through the normalized value (z) of λ, obtained by dividing lambda by its standard error. An association was considered significant at the 0.05 level if $|z| > 1.96$. Because of the large number of comparisons undertaken, the Bonferroni correction according to Rice (1989) has been used. The SPSS statistical package version 6.01 was used to fit log-linear models.

The homozygosity (j) at the *GBA* locus in a given population was determined by

$$j = \Sigma x_i^2$$

where x_i is the frequency of the ith allele (Guldberg et al., 1996). As the identification of GD mutations is not 100% in most populations studied, we have calculated maximum and minimum values of the homozygosity index, either considering all the unidentified alleles as a single one or defining the unknown alleles as having a frequency of 1/N, where N is the total number of mutant chromosomes studied.

RESULTS

Clinical Manifestations

The clinical characteristics of GD among 42 type I patients are summarized in Table 1. Patients are grouped according to genotype. The age of presentation is shown for all patients (if known), while the age at evaluation is only indicated for those patients whose age at first symptom presentation was unknown or for those with early manifestations of the disease. Phenotypes of patients I.1–I.26, documented previously (Cormand et al., 1995), are listed for comparison with the remaining patients and because additional clinical signs are indicated in some cases.

Splenomegaly was noted in all type I patients, except patient I.26, although his affected sibling presented with hepatosplenomegaly. At the age of 40, patient I.26 had been diagnosed of an atypical parkinsonian syndrome because of hypokinesia, rigidity and tremors affecting mainly the right side, and a poor response to conventional antiparkinson therapy. Two patients with early manifestations of GD in childhood (I.31 and I.38) had short stature, a sign that has been associated with massive spleen enlargement in children. Hepatomegaly was present in 23 out of the 42 type I patients (54.7%). Liver disease was encountered in patients I.4, I.27, and I.31 and was probably cause of death in patient I.32. Various degrees of bone involvement were found in 13 type I patients (31%). They showed widening of the distal femurs (Erlenmeyer flasks deformity) or hip osteoarthritis as the most frequent signs. In type I GD patients, thrombocytopenia was the most common cytopenia (15 patients, 35.7%); 5 patients had anemia and 9 patients had pancytopenia.

Table 2 shows the clinical findings of 11 patients with the neuronopathic forms of the disease. The time of onset ranged from birth to age 11 months in type II patients. Case II.2 was a fetus that was aborted following prenatal diagnosis. The parents had previously had an affected child, who was diagnosed for the first time at the age of 8 months with persistent emesis and rectal bleeding. Significant clinical signs were hepatosplenomegaly with abdominal pain, generalized eczema, and moderate thrombocytopenia. Microcephaly, deafness, and spasticity were also observed. Massive bleeding from birth was also present in patients II.7 and II.8. Case III.3 is the only individual alive among the type III patients so far analyzed. His neurological condition has not further deteriorated at the present age of 13, while splenomegaly has progressed with severe thrombocytopenia.

Mutation Analysis

We have analyzed 106 Spanish GD chromosomes by digestion with restriction enzymes, ASO hybridization, SSCP analysis, and sequencing, and we have identified 24 different mutations in the *GBA* gene that account for 95.3% of all Spanish GD alleles.

All the mutations identified and their genomic localization are shown in Figure 2. The genotypes of the patients included in this study are given in Tables 1 and 2.

Two amino acid substitutions, N370S and L444P, account for 66.1% of the total number of alleles, while the other 22 mutations are represented at frequencies lower than 5%. Mutation D409H (4.7%) is the third most common GD allele in this group of patients (Table 3). Three patients carry the Rec*Nci*I mutation, a crossover between the *GBA* gene and its closely linked pseudogene (Zimran et al., 1990b). This systematic screening revealed 14 novel mutations, including 8 amino acid substitutions (G113E [455G→A], R120W [475C→T], T134P [517 A→C], Y313H [1054 T→C], G389E [1283G→A], P391L [1289C→T], N392I [1292A→T], Y412H [1351T→C]), 3 stop codons (W(-4)X [108G→A],

TABLE 1. Patients and Genotypes: Type I Patients

Patient subtype/No.	Genotype	Age[a]	Clinical signs
I.2, I.7, I.9, I.12, I.17, I.36	N370S/N370S	27, 68, 4, 56, 58, 82	All S but not H; three asymptomatic; most very mild
I.4, I.10, I.14, I.16, I.19, I.20, I.21, I.23, I.25, I.34, I.37, I.39	N370S/L444P	2 (32), 33, 29, 25, 19, 48, 7 (31), 5, 24, inf (23), 60, 3	All S. 6 patients also H. 7 with pancytopenia, 4 with bone disease; one (I.39) asymptomatic at present age of 5 yr
I.13, I.30, I.31	N370S/RecNciI	28, 42, 13	I.13: SH, thrombocytopenia, cystalgia; I.30: S, thrombocytopenia, leukopenia, chronic diarrhea, skin pigmentation; I.31: SH, thrombocytopenia, leukopenia, stature and pubertal development delayed; portal hypertension; irregularity of right humerus
I.8, I.24	N370S/P391L	31, 27	Both: SH, epistaxis; I.8: pancytopenia, femur deformity. I.24: thrombocytopenia
I.1	N370S/?	7	S only
I.3	N370S/?	3	SH; macrocephaly
I.5	N370S/1451delAC	15	S, thrombocytopenia, anemia, bone pain
I.6	N370S/Y313H	10	SH, thrombocytopenia
I.11	N370S/1098insA	3 (9)	SH, leukopenia, splenectomy at 9 yr
I.15	N370S/R359Q	35	SH, epistaxis, easy bruising, femur deformity, conjunctival pingueculae
I.18	N370S/1263del55	5	SH, femur infarcts
I.26	N370S/500insT	47	No SH; atypical parkinson[b]
I.27	N370S/T134P	? (39)	SH, digestive hemorrhagia, ulcus gastroduodenal, hepatopathy, skin pigmentation, bone deformities
I.28	N370S/W(-4)X	4	SH, mild pancytopenia, epistaxis, bone crisis, femur deformity, skin pigmentation
I.29	N370S/G113E	7 m (4)	SH, anemia, bone disease
I.33	N370S/G202R	25	S, pancytopenia, bone deformity, aseptic necrosis
I.35	N370S/IVS5+1	? (24)	Data not available
I.38	N370S/G195E	8 (36)	SH, anemia, thrombocytopenia, stature development delayed, jaundice, skin pigmentation
I.40	N370S/Y412H	? (26)	S, thrombocytopenia, familial factor IX deficiency[c]
I.41	N370S/R257X	6	SH, pancytopenia
I.42	N370S/Q169X	? (87)	SH, epistaxis, pancytopenia
I.22	D409H/E326K	2.5	SH only (present age 14 yr)
I.32	?/?	? (76)	SH; hepatopathy, cardiomegaly, hip osteoarthritis, vitiligo, pale skin

S, splenomegaly; H, hepatomegaly; inf, infancy.
[a]At presentation (years). Age at evaluation is indicated in parentheses for patients with an early or unknown age of disease presentation.
[b]Affected brother (46 yr) with SH, thrombocytopenia, and pingueculae.
[c]Affected sibling (23 yr) with same symptoms plus bone disease.

Q169X [622C→T], R257X [886C→T]), two 1-bp insertion (500insT, 1098insA), and one donor splice-site mutation (IVS5+1G→T). Data on mutations R120W, Y313H, and 1098insA are reported elsewhere (Chabás et al., 1996; Cormand et al., 1996), and the rare G202R (721G→A), E326K (1093G→A), and R359Q (1193G→A) have already been detected in non-Spanish patients once before (Kawame et al., 1992; Beutler et al., 1994). Very recently, two of the mutations detected in this work, G195E [701 G→A] and 1451delAC, were described for the first time (Grace et al., 1997). It should be noted that the Spanish patient bearing the 1451delAC mutation, described by these authors, is our patient I.5 who received treatment at Mt. Sinai Hospital. Most of these new or rare mutations were found only once in this panel of patients. Only mutations P391L and G195E were found twice.

Screening of 80 normal chromosomes failed to detect any of the 24 mutations described in this study. After this systematic screening, five GD alleles remained unidentified. Three of them were present in heterozygosis with a known GD allele in patients I.1, I.3, and II.4 (Tables 1, 2), whereas no mutation was identified in patient I.32. Patient I.3 was initially genotyped as N370S/N370S in a previous study (Cormand et al., 1995), but further analysis of the parents showed that only the mother was a carrier of the mutation. Eight polymorphic microsatellite markers in different chromosomes were used to confirm paternity, and Southern blot analysis after *Bam*HI digestion (data not shown) showed a band pattern different from that of wild-type individuals, indicat-

TABLE 2. Patients and Genotypes: Type II and III Patients

Patient subtype/No.	Genotype	Age at presentation/death	Clinical signs
II.1	L444P/L444P	1 m/8.5m	SH, thrombocytopenia, anemia, seizures, head retroflection, hyperreflexia
II.7	L444P/L444P	2 m/3m	SH, growth retardation, thrombocytopenia, massive bleeding, coagulopathy, bronchoaspiration
II.8	L444P/L444P	birth/2.5 m	SH, low-weight neonate, anemia, thrombocytopenia, rectal bleeding, ascites, hepatocellular damage, cholestatic jaundice
II.2	L444P/G195E	Fetus	
II.3	L444P/R120W	5 m/9 m	SH, generalized hypertonia, neck hyperextension, opisthotonos, strabismus, osteoporosis, pulmonary fibrosis
II.4	G389E/?	6 m/10 m	SH, myoclonic seizures, hypertonia, psychomotor retardation
II.5	L444P/N392I	6 m/12 m	SH, hypertonia, opisthotonos, strabismus, head retroflection
II.6	D409H/R120W	11 m/20 m	SH, generalized spasticity, psychomotor retardation
III.1	L444P/L444P	3 yr/7.5 yr	SH, respiratory complications, bone pain, and fractures
III.2	D409H/D409H	?/19 yr	SH, epistaxis, strabismus, ophthalmoplegia, pes cavus, cardiovascular disease[a]
III.3	L444P/D409H	3 yr	SH, strabismus, ophthalmoplegia, thrombocytopenia (present age: 13 yr)

S, splenomegaly; H, hepatomegaly.
[a]One affected sibling without H (see Chabás et al., 1995, for further details).

ing that a deletion, or another type of rearrangement, could be the cause of this erroneous assignment, similar to the case described by Beutler and Gelbart (1994). Further analyses on this case are currently being undertaken.

Heterogeneity in the *GBA* Gene in Different Populations

In order to compare the mutation heterogeneity at the GBA locus between the Spanish and other populations, we used allele frequencies to calculate the homozygosity as in Gulberg et al. (1996) (Table 3). We chose those populations for which at least 70% of the mutated alleles were identified. Norrbottnian GD patients are all homozygous for the same mutation (L444P) (Dahl et al., 1990), giving a homozygosity value of 1. Ashkenazi Jewish patients are also quite homogeneous (0.50–0.59) due to the high frequency of mutations N370S and 84GG (Horowitz et al., 1993; Balicki and Beutler, 1995). The Spanish disease population has an intermediate value of 0.24, higher than most non-Jewish GD populations.

Linkage Disequilibrium Between the 6144A→G Intragenic Polymorphism and Mutations N370S and L444P

We analyzed the 6144A→G intragenic polymorphism in intron 9 of the GBA gene in all 53 Spanish GD patients. In addition, 44 nonaffected Spanish individuals were genotyped in order to determine the frequency of the two alleles in the Spanish population. The + allele accounts for 29.5% (26 of 88) of the chromosomes studied and the – allele for 70.5% (62 of 88), in agreement with previous reports (Sorge et al., 1985; Glenn et al., 1994). As the 12 polymorphic sites described within the GBA gene have been reported to be in complete linkage disequilibrium (Beutler et al., 1992b), we analyzed only one of them, the 6144A→G (*Hha*I) site.

Our data revealed that mutation N370S was always associated with the common – variant in these patients (46 of 46). Mutation L444P was found associated with both the + (15 of 24) and the – (9 of 24) alleles. However, there was a clear overrepresentation of L444P alleles in the context of the less frequent + allele. Significant linkage disequilibrium values were obtained in both cases (N370S: $D = 0.067$, $D' = 1$, $P < 0.001$; L444P: $D = 0.055$, $D' = 0.408$, $P < 0.005$).

Haplotype Analysis of N370S Chromosomes

In order to study identity by descent by shared haplotype analysis, five microsatellite markers closely linked to the GBA gene on chromosome 1q21 were analyzed in 24 chromosomes bearing the common N370S mutation. We studied 6 unrelated Spanish GD patients with genotype N370S/N370S (I.2, I.7, I.9, I.12, I.17, and I.36) and 3 heterozygous for N370S

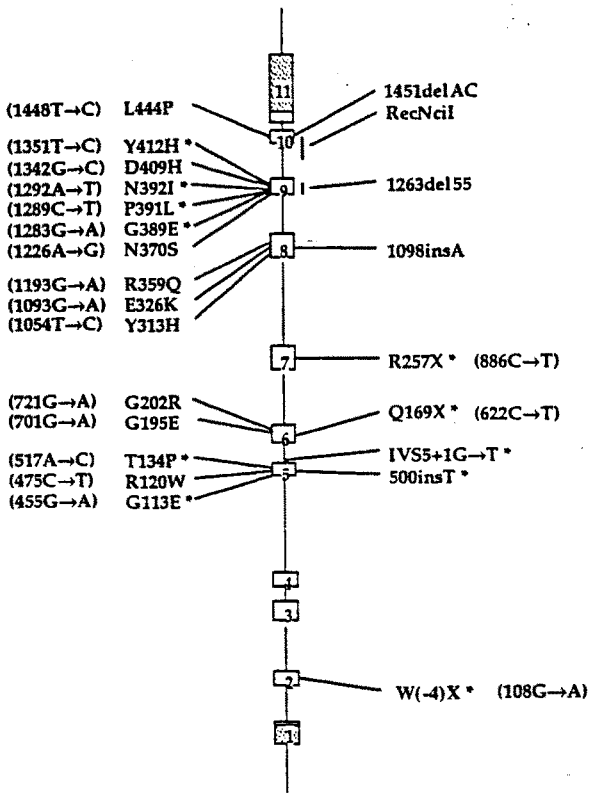

FIGURE 2. Schematic representation of the mutations identified in the β-glucocerebrosidase gene in Spanish Gaucher disease patients. Missense mutations are depicted on the left; gene/pseudogene recombinations, insertions, deletions, aberrant splice sites, and nonsense mutations are shown on the right. Novel mutations are indicated by an asterisk (*). *In brackets*, the cDNA position of the nucleotide change; *shaded boxes*, untranslated regions, and numbers correspond to exons.

(I.6, I.11, and I.18). Additionally, we analyzed 9 chromosomes, bearing the N370S allele, from Argentinian patients. Three of these chromosomes were found in two patients of Ashkenazi Jewish origin.

Full haplotypes for the five loci were established. One major haplotype, 1-4-3, for markers D1S2777, D1S2140, and D1S2721, respectively, was present in 16 of 24 (66.7%) of the N370S chromosomes analyzed (Table 4). Eight chromosomes, belonging to individuals I.6, I.11, I.18, I.36, and to three of the Argentinian patients, bore other allelic combinations: 1-4-7 (I.6), 1-4-6 (I.11), 1-4-1 (I.18), 6/1-1/4-2/1 (I.36, phase unknown) and 1-3-8, 1-6-1, 1-4-1 (Argentinian patients). Interestingly, the Spanish consensus haplotype is identical to the haplotype present in the 3 chromosomes of known Ashkenazi Jewish origin.

In order to measure the degree of allelic association between each marker and the N370S mutation, we applied hierarchical log-linear models. Table 4 summarizes the results of this analysis. Significant values of association ($|z| > 1.96$) were obtained for alleles 1, 4, 3, and 5 of markers D1S2777, D1S2140, D1S2721, and D1S2624, respectively.

DISCUSSION

Mutation Analysis

The prevalence of GD is at its highest in the Ashkenazi Jewish population, thus most studies have focused on this ethnic group. Most of the studies of mutations causing GD carried out on non-Jewish patients were performed on individuals of diverse ethnic (Beutler and Gelbart, 1993) or nonspecified origin (Horowitz et al., 1993). By contrast, the studies on specific non-Jewish populations were limited to the analysis of a relatively small group of previously described mutations (Walley et al., 1993; Cormand et al., 1995; Michelakakis et al., 1995; Amaral et al., 1996; Tylki-Szymanska et al., 1996; le Coutre et al., 1997). Here we report an exhaustive search for the mutations present among Spanish GD patients, which allowed the identification of 95% of the mutant alleles in the 53 patients analysed. Mutations N370S, L444P, D409H, and RecNciI account for around 73% of the total number of GD chromosomes, while the remaining 27% is accounted for by at least 20 different mutations, including 14 novel, three of them already reported by us (Chabás et al., 1996; Cormand et al., 1996). After extensive SSCP screening covering the 11 exons of the *GBA* gene, all the splice sites and part of the 5′ and 3′ untranslated regions, only 5 mutant alleles remained unidentified.

Five of the 11 novel variants described in this paper are undoubtedly disease-causing mutations, as they create a frameshift (500insT), premature stop codons (W(-4)X, Q169X, R257X), or give rise to abnormal splicing (IVS5+1G→T). Mutation IVS5+1G→T was the only noncoding alteration found. Although the effect of this substitution in the first nucleotide of intron 5 was not studied in the patient RNA because no sample was available, this position is 100% conserved in the vertebrate donor splice-site consensus (Padgett et al., 1986). The other 6 new mutations are amino acid substitutions, and several findings strongly suggest that they are also disease-causing mutations: (1) after exhaustive examination of the *GBA* gene, no other mutation was found in any of the patients; (2) the changes were not present in 80 normal chromosomes analyzed; (3) all the amino acid residues involved are conserved in murine and human GBA, indicating functional/structural relevance; and (4) prediction of protein secondary structure indicates that all mutations except for Y412H may produce conformational changes in the mature enzyme. Moreover, all these

TABLE 3. Prevalence (%) of the Four Most Common *GBA* Mutations in Different Populations and Homozygosity (Σx_i^2) at the *GBA* Locus as a Measure of Genetic Heterogeneity

Population	Σx_i^2 [a]	N370S	84GG	IVS2+1	L444P	D409H	RecNciI[b]	R463C	G377S	No. of chromosomes	% mutation detection
Norrbottnian[1]	1.00 (1.00)	—	—	—	100	—	—	—	—	22	100
Ashkenazi Jewish[2]	0.59 (0.59)	76.45	11.96	2.17	3.26	—	—	—	—	276	99.3
Ashkenazi Jewish[3]	0.50 (0.51)	69.77	10.17	2.26	4.24	—	—	—	—	354	90.4
Portuguese[4]	0.31 (0.33)	53.70	—	—	12.96	—	—	3.70	7.41	54	87.0
Spanish[5]	0.24 (0.25)	43.40	—	—	22.64	4.72	2.83	—	—	106	95.3
Non-Jewish[3]	0.19 (0.20)	22.86	—	—	31.43	5.00	7.80	—	—	240	89.2
Australasian[6,7]	0.17 (0.25)	25.00	3.57	—	30.36	—	7.14	—	—	56	69.6
Non-Jewish[2]	0.16 (0.21)	30.00	—	—	30.00	—	4.17	4.17	—	140	77.1

[a]Less common and uncharacterized alleles were counted as different alleles or as one single allele (the latter shown in brackets).
[b]Including cases described as XOVR.
References: [1]Dahl et al. (1990; [2]Balicki and Beutler (1995); [3]Horowitz et al. (1993); [4]Amaral et al. (1996); [5]present study; [6]Lewis et al. (1994); [7]Nelson et al. (1995).

TABLE 4. Allelic Association Between the N370S Mutation and Markers on Chromosome 1q21

Marker	No. of alleles/ heterozygosity[a]	Associated allele		% of N370S chromosomes	% of control chromosomes[c]	λ	|z|	P*
		Designation[b]	Sequence					
D1S305	9/0.86	7	$(AC)_{17}$	12.5% (3/24)	3.6% (2/56)	0.70	1.72	<0.09
D1S2777	9/0.57	1	$(AC)_{16}$	95.8% (23/24)	57.1% (32/56)	0.57	2.13	<0.04
D1S2140	7/0.71	4	$(AT)_6...(TATC)_{11}$	87.5% (21/24)	30.4% (17/56)	0.83	3.16	<0.002
D1S2721	8/0.65	3	$(AC)_{18}$	66.7% (16/24)	23.1% (12/52)	0.70	2.69	<0.008
D1S2624	5/0.71	5	$(AC)_{20}$	16.7% (4/24)	1.8% (1/56)	0.92	2.24	<0.03

[a]Dib et al. (1996).
[b]According to CEPH-Généthon nomenclature.
[c]CEPH-Généthon data.
*All values are significant at $P < 0.05$, except for allele 7 of marker D1S305.

substitutions involve a change in the polarity or charge of the residue (G113E, T134P, G389E, N392I, Y412H) or are structurally disruptive (P391L). However, further characterization of the products expressed from each mutant allele is needed to confirm these assumptions.

Our results show that the 15 mutations leading to amino acid substitutions are mostly confined to a few exons, while nonsense, frameshift and splice-site mutations are widely distributed throughout the gene (Fig. 2). The clustering of naturally occurring missense mutations in exons 8 and 9 (9 of 15) and in exons 5 and 6 (5 of 15) of the GBA gene is consistent with previous data (Beutler and Gelbart, 1996), and confirms the important structural/functional roles of these regions, as deduced from biochemical analyses (Grace et al., 1990, 1994; Ohashi et al., 1991).

In order to enhance the sensitivity of the SSCP method, we assayed a battery of different conditions such as polyacrylamide and glycerol concentration in the gel and running temperature, and we chose the best four combinations for each amplified sequence. The importance of varying the experimental parameters is demonstrated by the finding that some of the mutations described here were detected in only one or two of the four conditions assayed. The intrinsic limitations of the SSCP technique or the presence of mutations outside the regions analyzed may explain why four mutated alleles were not detected (the fifth missing allele is the uncharacterized deletion of patient I.3). Complete sequencing of the GBA gene or the cDNA from patients bearing these unidentified alleles may reveal the underlying mutations. Interestingly, patient I.32, with clear symptoms of GD, is the only one with two unidentified mutations, and this leaves open the unlikely possibility of a different genetic cause for the disease. The low β-glucocerebrosidase activity rules out the prosaposin gene as responsible for GD.

The complete mutation analysis performed on the Spanish GD patients provides interesting data for molecular diagnosis. The detection of the most prevalent mutations in this population, N370S, L444P, D409H, and Rec*Nci*I, allows full identification of nearly half of the genotypes (47.2%). Moreover, another 35.8% is partially covered by this analysis, as they are heterozygous for N370S, which provides essential information for the prognosis of the disease.

Genotype/Phenotype Correlations

The large heterogeneity observed in clinical manifestations of GD results, in part, from the large number of mutations in the GBA gene. However, many unrelated or related patients with the same genotype have been described with significantly distinct clinical features, indicating that other factors may play an important role.

The presence of the N370S mutation, either in homozygosity or in heterozygosity, always precludes development of neurological manifestations in our patients, in agreement with previous data (Beutler and Grabowski, 1995). However, the phenotypic expression is highly variable. Those with genotype N370S/N370S are the least severe, including three asymptomatic individuals (see Table 1). The age of onset varies within this group from 4 to 82 years. Patients with genotype N370S/L444P present more severe symptoms and the average age of onset (23.2 ± 18.0 years) is significantly lower than that of patients who are homozygous for N370S (49.2 ± 26.1). Phenotypic variation is also observed among the three patients with genotype N370S/rec*Nci*I and between the two patients with N370S/P391L.

It is well established that homozygosity for the L444P mutation causes severe disease with neurological involvement, but the clinical expression ranges from the juvenile form or type III to the infantile neuronopathic form or type II (Beutler, 1995). Interestingly, some Japanese L444P/L444P patients have been found among the non-neuronopathic form of

GD (Ida et al., 1995). Four unrelated Spanish GD patients have been genotyped as L444P/L444P (Table 2). Three of these patients represent the type II of the disease, and the other was classified as type III.

Recently the association between the D409H/D409H genotype and a unique clinical presentation consisting of cardiovascular abnormalities and oculomotor apraxia (Abrahamov et al., 1995; Uyama et al., 1997) has been reported. This association is illustrated in our group of patients by individual III.2 and her two sisters, described in detail elsewhere (Chabás et al., 1995).

Only two of the 24 mutations described in this study could be classified as mild: the most prevalent N370S and E326K. Mutations 1451delAC, 1263del55, 500insT, 1098insA, W(-4)X, Q169X, R257X, and IVS5G→T belong to the "lethal" group, and mutations R120W, G195E, G389E, N392I, D409H, and L444P to the "severe" group, according to the classification by Beutler et al. (1994). Eight mutations could not be assigned to any group because they were found together with the common mild N370S mutation, which is always associated with a type I phenotype.

Linkage Disequilibrium, Haplotype, and Population Analyses

From our data it is reasonable to conclude that the L444P mutation arose more than once in this group of patients, since it appears in the context of both the + and – variants of the intragenic polymorphism. It is significant that this mutation is present in the normal pseudogene sequence, suggesting that gene conversion events could be responsible for these recurrent changes. However, significant linkage disequilibrium was observed between this mutation and the less common + allele ($D = 0.055$, $D' = 0.408$, $P < 0.005$), suggesting expansion of an ancestral allele.

The N370S allele is invariably associated with the – allele in our group of patients, in agreement with previous data from the Ashkenazi Jews (Beutler et al., 1992b) and from other populations (Amaral et al., 1996). These results are consistent with a common origin for the panethnic N370S *GBA* allele. However, because the – allele is about twice as common in the general population, as the + (Zimran et al., 1990a), recurrent mutations cannot be formally ruled out from these data.

We have recently mapped the *GBA* gene genetically (Cormand et al., 1997b). The analysis of three microsatellite markers, known to be very close to the gene, in 24 N370S alleles from Spanish and Argentinian patients, showed that one major haplotype was present in 66.7% of the chromosomes. The same haplotype was found in the three Ashkenazi Jewish chromosomes analyzed bearing N370S. It would be interesting to study more chromosomes from the Jewish population to evaluate the possibility of a common origin for the Spanish and the Ashkenazi Jewish N370S mutation.

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PATOLOGIA MOLECULAR DE LA MALALTIA DE GAUCHER

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INTRODUCCIÓ

La malaltia de Gaucher és un trastorn degut a la disfunció del sistema lisosòmic. Les malalties lisosòmiques es classifiquen segons la via metabòlica afectada i la naturalesa química del substrat acumulat. En el cas de la malaltia de Gaucher la deficiència afecta el catabolisme dels esfingolípids i és, per tant, una esfingolipidosi. És l'esfingolipidosi prevalent.

La simptomatologia associada a la malaltia de Gaucher presenta els següents trets distintius: increment de la mida del fetge i de la melsa (hepatoesplenomegàlia); substitució progressiva de la medul·la òssia per macròfags carregats de lípids (cèl·lules de Gaucher) que comprometen la producció d'eritròcits i de plaquetes (anèmia, trombocitopènia); degeneració osteolítica de l'esquelet que pot ocasionar crisis òssies, i en una minoria de pacients una degeneració progressiva del sistema nerviós central.

Les manifestacions clíniques de la malaltia de Gaucher són molt heterogènies. S'ha classificat en tres subtipus clínics d'acord amb l'absència (tipus I) o presència i gravetat (tipus II i III) de les alteracions neurològiques (Beutler i Grabowski, 1995).

El tipus I, forma no neuropàtica (MIM 230800), és el més freqüent, amb una incidència d'entre 1/40.000 i 1/60.000 en la població general. La incidència s'eleva a d'entre 1/400 i 1/600 en la població jueva asquenasita. Es caracteritza per l'absència d'afectació del sistema nerviós central i la seva presentació és molt variable tant pel que fa a l'edat d'aparició dels primers símptomes com a la gravetat i progressió de la malaltia.

El tipus II, forma neuropàtica aguda (MIM 230900), té una incidència de 1/100.000 naixements, sense predomini ètnic. És la forma més greu a causa de l'acumulació neurovisceral ràpida de lípids que provoca la mort durant els dos primers anys de vida. Les manifestacions neurològiques

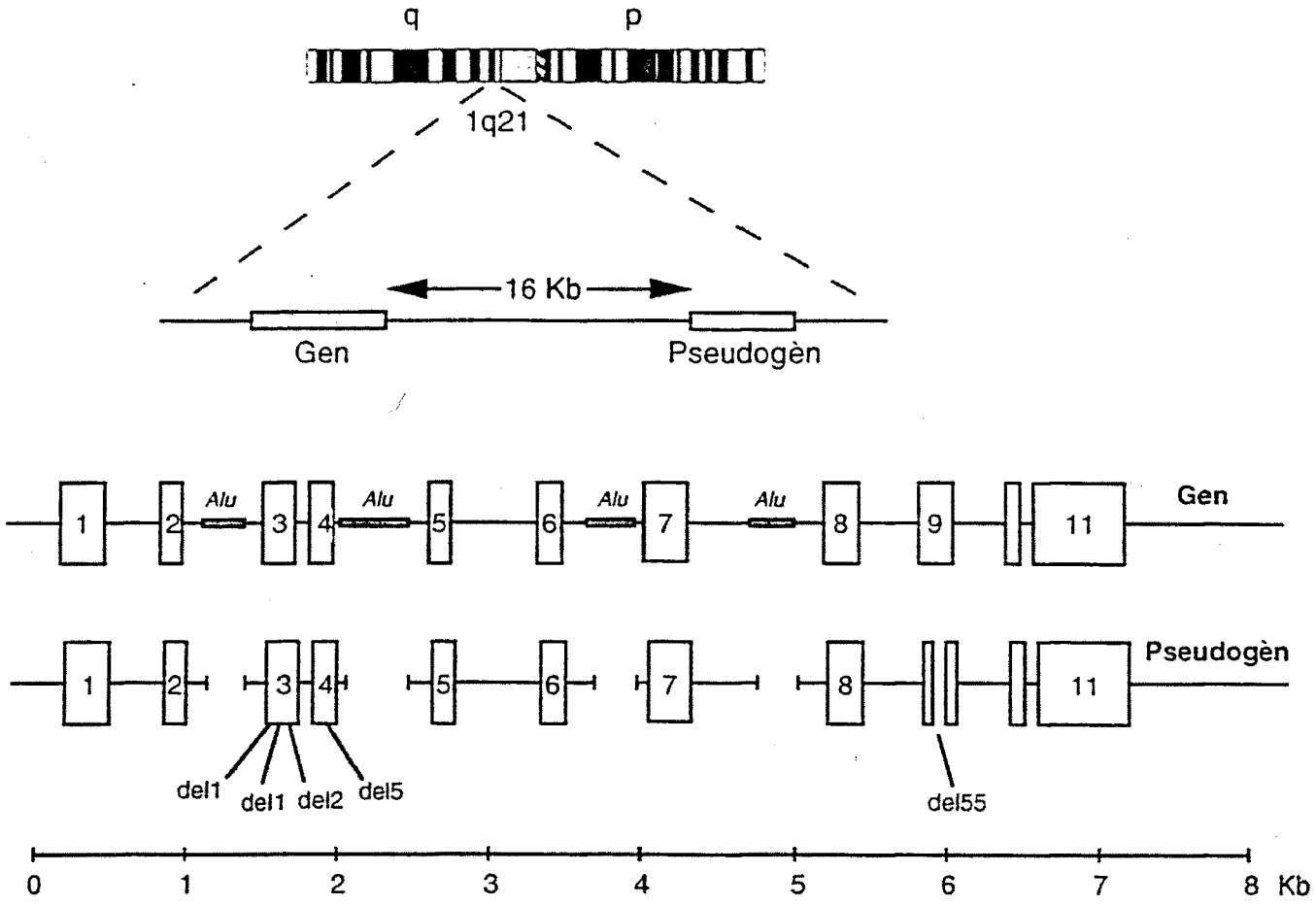

FIGURA 1. Localització cromosòmica i estructura del gen de la β-glucocerebrosidasa (GBA) i del seu pseudogèn.

inicials són generalment anomalies oculomotores.

El tipus III, forma neuropàtica subaguda (MIM 231000), es manifesta amb una gravetat intermèdia entre els tipus I i II. De progressió menys ràpida i amb afectació neurològica més tardana, permet la supervivència fins a l'edat adulta. La seva incidència varia entre 1/50.000 i 1/100.000 naixements i, tot i que no presenta predomini ètnic, és particularment prevalent a la regió de Norrbottnia, al nord de Suècia.

La malaltia de Gaucher presenta una herència autosòmica recessiva i és deguda en la major part dels casos a una deficiència en l'enzim β-glucocerebrosidasa (Brady *et al.*, 1965). La β-glucocerebrosidasa és una hidrolasa àcida lisosòmica que catalitza la hidròlisi de glucosilceramida a ceramida i glucosa en presència d'una proteïna activadora anomenada saposina C o SAP-C. El mal funcionament de la β-glucocerebrosidasa provoca l'acumulació de glucosilceramida (o glucocerebròsid) a l'interior dels lisosomes dels macròfags en el sistema reticuloendotelial, sobretot a la melsa, el fetge i la medul·la òssia.

El gen que codifica la β-glucocerebrosidasa (GBA), una glicoproteïna de membrana de 497 aminoàcids i uns 65 kDa, ha estat clonat i localitzat en el braç llarg del cromosoma 1, a 1q21 (Ginns *et al.*, 1985). Té onze exons i deu introns i cobreix una regió genòmica d'unes 7 kb. Els introns 2, 4, 6 i 7 contenen seqüències *Alu* (figura 1). S'ha identificat un pseudogèn a unes setze kilobases en direcció 3' respecte al gen funcional, que es transcriu però no es tradueix a proteïna. La

seva identitat amb el gen pel que fa a seqüència és del 96 % (Horowitz *et al.*, 1989). El pseudogèn té una estructura d'exons i introns semblant al gen però no conté les seqüències *Alu* i presenta diverses mutacions que impossibiliten la síntesi d'una proteïna funcional (figura 1).

S'han descrit tretze polimorfismes en el gen *GBA*, dotze en zona no codificant que estan en desequilibri de lligament entre ells i donen lloc a dos haplotips majoritaris (Beutler *et al.*, 1992).

En la majoria dels casos la malaltia de Gaucher és deguda a mutacions en el gen *GBA*. Només en dos malalts s'ha trobat el defecte en el gen que codifica la proteïna activadora SAP-C (Christomanou *et al.*, 1986; 1989), situat a 10q21-22.

S'han descrit fins ara més de cent mutacions en el gen de la glucocerebrosidasa que donen lloc a la malaltia de Gaucher. Aquestes alteracions inclouen mutacions puntuals de canvi d'aminoàcid i sense sentit, insercions, delecions, mutacions de la maduració per tall i unió i reordenaments entre el gen i el pseudogèn (Balicki i Beutler, 1995; Beutler i Gelbart, 1997). Aquests reordenaments originen al·lels complexos que contenen diverses mutacions com a resultat d'entrecreuaments desiguals o de fenòmens de conversió gènica.

De les moltes mutacions que s'han identificat en el gen *GBA*, poques tenen una prevalença elevada, però la seva freqüència relativa varia segons el grup ètnic. En els jueus asquenasites, la mutació N370S, un canvi d'asparagina a serina en la posició 370 de la proteïna, és la més freqüent i està present en un 68-77 % dels al·lels mutats. La segona mutació més freqüent és una inserció d'una guanina en la posició 84 del cDNA (84GG), que representa un 10-13 %. Només quatre mutacions (N370S, 84GG, L444P i IVS2+1) expliquen el 89-96 % dels defectes moleculars responsables de la malaltia en aquesta població (Beutler *et al.*, 1992; Horowitz *et al.*, 1993; Sibille et al., 1993).

En les poblacions no jueves d'origen europeu les mutacions més freqüents són les substitucions N370S (23-54 %) i L444P (13-57 %) (Beutler *et al.*, 1992; Horowitz *et al.*, 1993; Dahl *et al.*, 1990; Tuteja *et al.*, 1993; Walley *et al.*, 1993; Cormand *et al.*, 1995; Michelakakis *et al.*, 1995; Amaral *et al.*, 1996; Tylki-Szymanska *et al.*, 1996).

S'han observat correlacions clares entre algunes mutacions i l'expressió fenotípica de la malaltia. Això té importància pràctica de cara al consell genètic i a la teràpia. La presència de la mutació N370S, tant en homozigosi com en heterozigosi, va invariablement associada a un diagnòstic no neuronopàtic i, per tant, al tipus I de la malaltia. En canvi, l'al·lel L444P (en absència de N370S) es correlaciona amb les formes neuronopàtiques de la malaltia (Tsuji *et al.*, 1988; Theophilus *et al.*, 1989). Recentment s'ha observat una associació entre el genotip D409H/D409H i un fenotip caracteritzat per calcificacions cardiovasculars i apraxia oculomotora (Chabás *et al.*, 1995; Abrahamov *et al.*, 1995).

S'han desenvolupat diverses estratègies terapèutiques per a la malaltia de Gaucher, a causa de l'existència d'una forma no neuropàtica de gran prevalença. El trasplantament de moll de l'os s'ha substituït per la terapèutica enzimàtica de substitució, que consisteix en la infusió de glucocerebrosidasa modificada (ceredasa) dirigida als macròfags (Barton *et al.*, 1991). Aquesta terapèutica fou introduïda l'any 1991 i actualment són ja més de mil les persones sotmeses a terapèutica de substitució enzimàtica al món. Si bé la majoria d'aquests pacients ha experimentat una clara millora en molts dels símptomes, el tractament és extremadament car i comporta una dependència de les infusions de per vida. La terapèutica gènica constitueix una nova estratègia per al tracta-

ment de la malaltia de Gaucher ja que les cèl·lules principalment involucrades són els macròfags, que deriven de les cèl·lules mare hematopoètiques. Actualment s'estan duent a terme proves clíniques inicials seguint els primers protocols aprovats (Dunbar *et al.*, 1996).

En aquest capítol descrivim alguns aspectes del treball realitzat pel nostre grup de recerca sobre la malaltia de Gaucher: la identificació de les mutacions en pacients espanyols (i també en un grup de pacients argentins i italians), l'estudi de correlacions genotip-fenotip, el mapatge genètic dels gens *GBA* i *PSAP*, el disseny d'una estratègia de diagnòstic molecular de la malaltia i la determinació del possible haplotip ancestral de la mutació N370S. Els malalts han estat diagnosticats de malaltia de Gaucher mitjançant l'anàlisi prèvia de l'activitat glucocerebrosidasa en cèl·lules com leucòcits o fibroblasts cultivats. El diagnòstic dels malalts espanyols es realitza a l'Institut de Bioquímica Clínica, que rep les mostres procedents de diferents hospitals d'arreu d'Espanya.

DETECCIÓ DE LES MUTACIONS RESPONSABLES DE LA MALALTIA DE GAUCHER

Quan vam iniciar el nostre treball no hi havia dades de mutacions responsables de la malaltia de Gaucher en la població espanyola, i la informació sobre altres poblacions no jueves era pràcticament inexistent. S'havien descrit mutacions en pacients no jueus, però no s'havien realitzat anàlisis exhaustives en poblacions específiques. El coneixement de les mutacions particulars d'una població és una eina molt important per dissenyar estratègies de diagnòstic molecular de la malaltia.

El nostre primer treball va consistir en l'anàlisi de la presència en pacients espanyols de mutacions prevalents descrites amb anterioritat en altres poblacions (Cormand *et al.*, 1995), mitjançant amplificació per PCR i digestió amb un enzim de restricció o bé utilitzant la tècnica d'hibridació amb oligonucleòtids específics d'al·lel (ASOH). Posteriorment hem analitzat exhaustivament la regió codificant del gen, tots el llocs de *splicing*, part dels introns i regions a 5' i a 3' del gen. Per això hem amplificat aquestes regions mitjançant PCR i a continuació hem utilitzat la tècnica de SSCP que permet detectar canvis puntuals en fragments curts de DNA. Aquest estudi ens ha permès identificar la mutació en cent-un de cent-sis al·lels mutats, que representen més del 95 % del cromosomes estudiats (figura 2). En total hem trobat vint-i-quatre mutacions diferents, catorze de les quals no havien estat mai descrites (Cormand *et al.*, 1996; 1998*a*; Chabás *et al.*, 1996).

Aquests darrers anys s'han realitzat estudis sobre altres poblacions, com ara l'anglesa (Walley *et al.*, 1993), la portuguesa (Amaral *et al.*, 1996), l' australiana (Lewis *et al.*, 1994; Nelson *et al.*, 1995), la grega (Michelakakis *et al.*, 1995), la polonesa (Tylki-

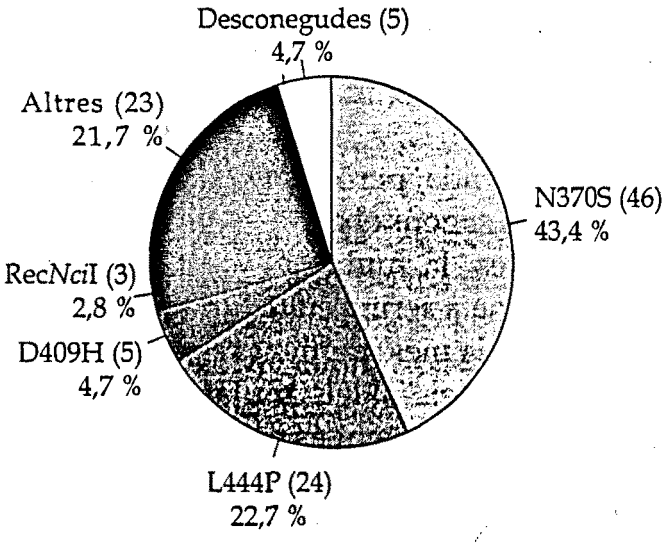

FIGURA 2. Freqüència relativa de les mutacions de la β-glucocerebrosidasa en cinquanta-tres pacients de Gaucher espanyols. Entre parèntesi s'indica el nombre d'al·lels.

Szymanska *et al.*, 1996) i l'alemanya (Lecoutre *et al.*, 1997). Aquests estudis, però, s'han limitat a l'anàlisi d'algunes mutacions prevalents prèviament descrites. Dos fets destaquen en la comparació de les freqüències de les mutacions entre la població espanyola i altres poblacions: per una banda, la mutació prevalent a l'Estat espanyol és la N370S (43,4 %), la mateixa que entre els jueus asquenasites (i també a Portugal), mentre que en altres poblacions aquest canvi és menys freqüent, per exemple un 27 % a Anglaterra o un 25 % a Austràlia. És per això que vam decidir estudiar el possible origen comú d'aquesta mutació en la població espanyola i en la jueva (veure més avall). L'altre fet a destacar és que la tercera mutació més freqüent a Espanya és la D409H, que és molt menys habitual en altres poblacions.

Recentment hem realitzat l'estudi d'una població de malalts de Gaucher d'Argentina (Cormand *et al.*, 1998*b*). Hem analitzat trenta-un pacients no emparentats i hem identificat la mutació responsable de la malaltia en cinquanta-vuit dels seixanta-dos al·lels mutats (94 %) mitjançant una metodologia similar a la utilitzada amb els pacients espanyols. La mutació N370S és

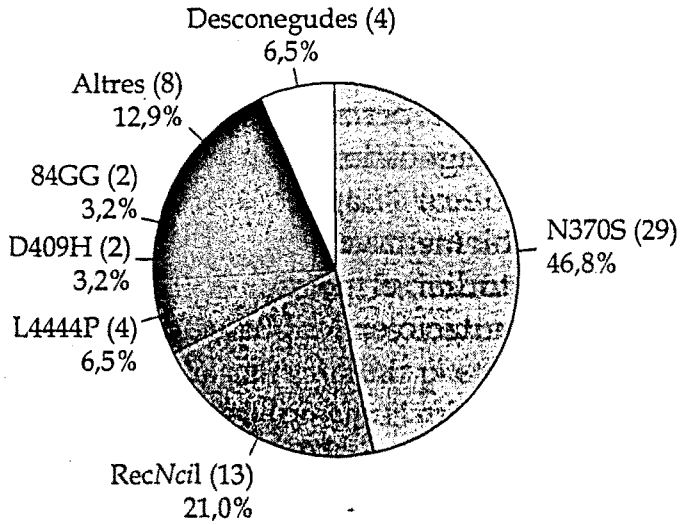

FIGURA 3. Freqüència relativa de les mutacions de la β-glucocerebrosidasa en trenta-un pacients de Gaucher procedents d'Argentina. Entre parèntesi s'indica el nombre d'al·lels.

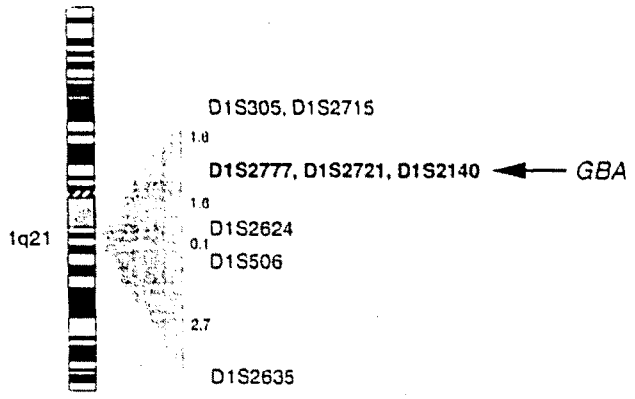

FIGURA 4. Localització genètica i citogenètica del gen de la β-glucocerebrosidasa (*GBA*) en el cromosoma 1. En negreta s'indiquen els marcadors tipus microsatèl·lit que es troben a 0 cM del gen. S'inclouen també altres marcadors propers i les distàncies relatives en cM.

també la més freqüent en aquesta població, però en aquest cas va seguida de l'al·lel recombinant anomenat RecNciI (figura 3). Aquesta mutació, originada a partir del pseudogèn, s'ha analitzat en detall tal com es descriu més avall. En els pacients argentins hem identificat tretze mutacions diferents, incloent-hi tres canvis descrits per primera vegada.

CORRELACIÓ GENOTIP-FENOTIP

El coneixement de les mutacions presents en cada pacient pot aportar una informació molt important per al diagnòstic prenatal, per al consell genètic i per al tractament. No obstant això, en molts casos no hi ha una correlació clara entre el genotip i el fenotip. En la malaltia de Gaucher l'única correlació ben establerta és l'associació entre la presència de la mutació N370S, en un o en els dos al·lels, i el tipus I de la malaltia, és a dir, les formes no neuronopàtiques. Beutler *et al.* (1994) han classificat les mutacions en «lleus», quan no hi ha afectació neurològica (el principal exemple és el canvi N370S); «greus», quan produeixen neuropatia, i «letals», quan no s'han trobat mai en homozigosi i no produeixen proteïna fun-

cional. Gairebé tots els pacients del tipus I tenen la mutació N370S. Per això es molt difícil identificar noves mutacions lleus.

Nosaltres hem identificat una mutació lleu en la població espanyola, la substitució aminoacídica E326K, present en un pacient de catorze anys sense neuropatia i amb el genotip E326K/D409H (Chabás *et al.*, 1996). La mutació D409H és greu i per tant el canvi E326K ha de ser el responsable de l'absència d'afectació neurològica. També hem identificat dues noves mutacions lleus en analitzar dos pacients italians (Cormand *et al.*, 1997*a*). Els dos pacients són del tipus I i homozigots per mutacions no descrites prèviament, I402T i V375L.

La novetat més interessant referent a la correlació genotip-fenotip que vam trobar fou el cas de tres germanes amb el genotip D409H/D409H que presentaven un fenotip particular amb calcificacions vasculars i moderada visceromegàlia (Chabás *et al.*, 1995). Poc després, altres autors varen publicar la presència de símptomes similars en pacients homozigots per a la mutació D409H, la qual cosa va reforçar aquesta nova correlació genotip-fenotip (Abrahamov *et al.*, 1995; Beutler *et al.*, 1995; Uyama *et al.*, 1997). Recentment hem identificat un nou pacient homozigot per la mutació D409H que té només dos mesos i que està ja rebent tractament de substitució enzimàtica (Chabás *et al.*, 1998). En aquest cas es podrà avaluar la prevenció de les calcificacions cardíaques pel tractament primerenc si assumim que el pacient hauria desenvolupat aquest símptoma com tots els altres malalts amb el genotip D409H/D409H descrits fins ara.

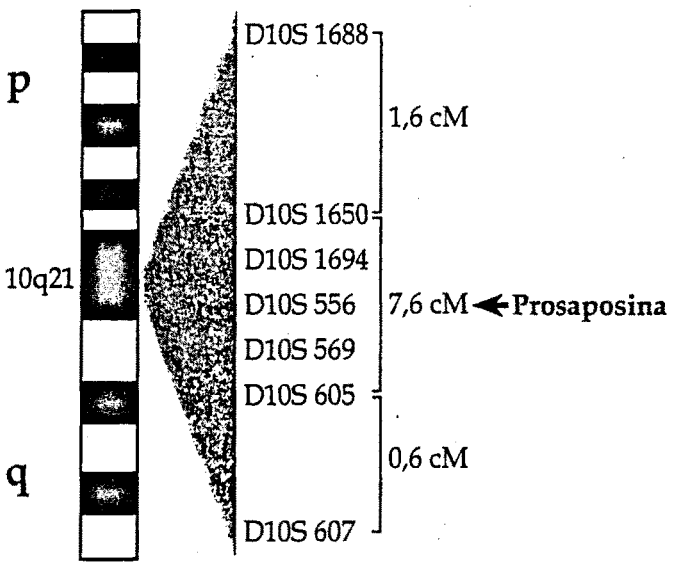

FIGURA 5. Localització genètica i citogenètica del gen de la prosaposina (*PSAP*) en el cromosoma 10. S'indica la posició del gen en relació a marcadors tipus microsatèl·lit i les distàncies relatives en cM.

MAPATGE DELS GENS *GBA* I *PSAP*

S'havia localitzat el gen *GBA* al cromosoma 1q21 mitjançant hibridació *in situ* (Ginns *et al.*, 1985), però no s'en coneixia la localització en relació a marcadors de tipus microsatèl·lit altament polimòrfics. Amb l'objectiu de fer el mapatge fi del gen, hem realitzat l'anàlisi de lligament de dos punts i multipuntual entre un polimorfisme intragènic i diversos microsatèl·lits del braç llarg del cromosoma 1 (Cormand *et al.*, 1997*b*). Hem mapat també el gen *PSAP* que codifica la prosaposina, precursora de la saposina C, i que és el responsable de la malaltia de Gaucher en un nombre reduït de casos. Els resultats del mapatge d'aquests dos gens es mostren en les figures 4 i 5. El mapatge del gen *GBA* ens ha permès dissenyar una estratègia de diagnòstic molecular indirecte, i també identificar l'haplotip ancestral en el qual probablement va tenir lloc la mutació N370S i estudiar el possible origen comú d'aquesta mutació en les poblacions espanyola i jueva.

DIAGNÒSTIC MOLECULAR INDIRECTE

A partir de l'estudi de l'espectre de les mutacions responsables de la malaltia de

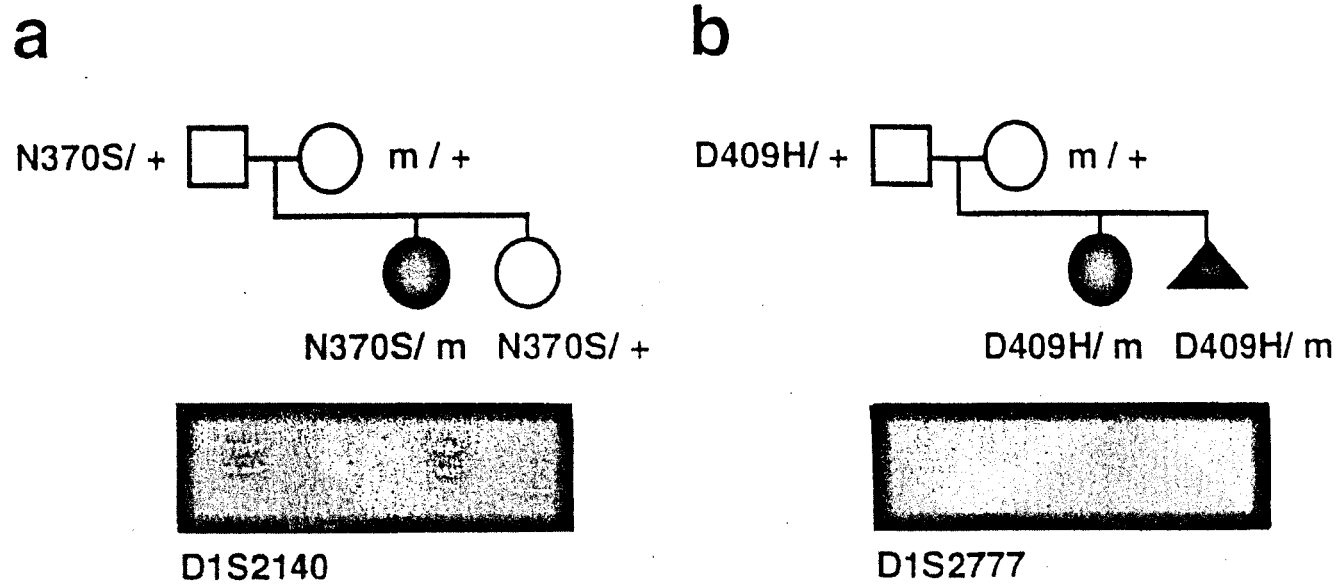

FIGURA 6. Anàlisi de cosegregació de la malaltia de Gaucher amb marcadors tipus microsatèl·lit del cromosoma 1q21 en dues famílies (a i b). Els productes de l'amplificació per PCR s'han separat en un gel de poliacrilamida al 6 % no desnaturalitzant i s'han tenyit amb bromur d'etidi. + = al·lel normal; m = al·lel amb una mutació desconeguda.

Gaucher en la població espanyola, l'Institut de Bioquímica Clínica, com a centre d'abast nacional per al diagnòstic d'aquesta malaltia lisosòmica, ha incorporat de manera rutinària la detecció molecular de les tres mutacions més freqüents (N370S, L444P i D409H). Si algun dels al·lels és portador d'una mutació diferent d'aquestes, s'ha de fer una anàlisi exhaustiva de tota la regió codificant per identificar la mutació mitjançant les tècniques que s'han descrit més amunt. Aquesta anàlisi no té cabuda dins de la rutina d'un laboratori de diagnòstic a causa del seu elevat cost, tant econòmic com de temps. Per això hem dissenyat una estratègia de diagnòstic molecular indirecte que consisteix a analitzar les tres mutaciones més freqüents i, si cal, estudiar posteriorment alguns dels marcadors tipus microsatèl·lit propers al gen per fer el diagnòstic d'afectat, portador o sa (Cormand *et al.*, 1998*c*). Un exemple d'aquest tipus d'anàlisi es mostra a la figura 6.

MUTACIONS ÚNIQUES O RECURRENTS? HAPLOTIP ANCESTRAL

Un element molt important per conèixer la història natural d'una mutació és saber si es tracta d'un esdeveniment que ha tingut lloc una única vegada (o poques vegades) o si, contràriament, es tracta d'un fet que es produeix amb una relativa freqüència. En el primer cas, una regió genòmica més o menys extensa al voltant de la mutació ha de ser idèntica en tots els cromosomes portadors, perquè l'alteració és heretada d'un ancestre comú. Aquesta identitat es detecta per l'existència de desequilibri de lligament entre la mutació i marcadors polimòrfics propers al gen, que portarien majoritàriament al·lels presents en el cromosoma original en què es produí el canvi.

Mitjançant l'anàlisi dels cromosomes que porten la mutació N370S hem pogut confirmar que aquesta mutació té un origen únic a la població espanyola i hem pogut reconstruir el probable haplotip ancestral

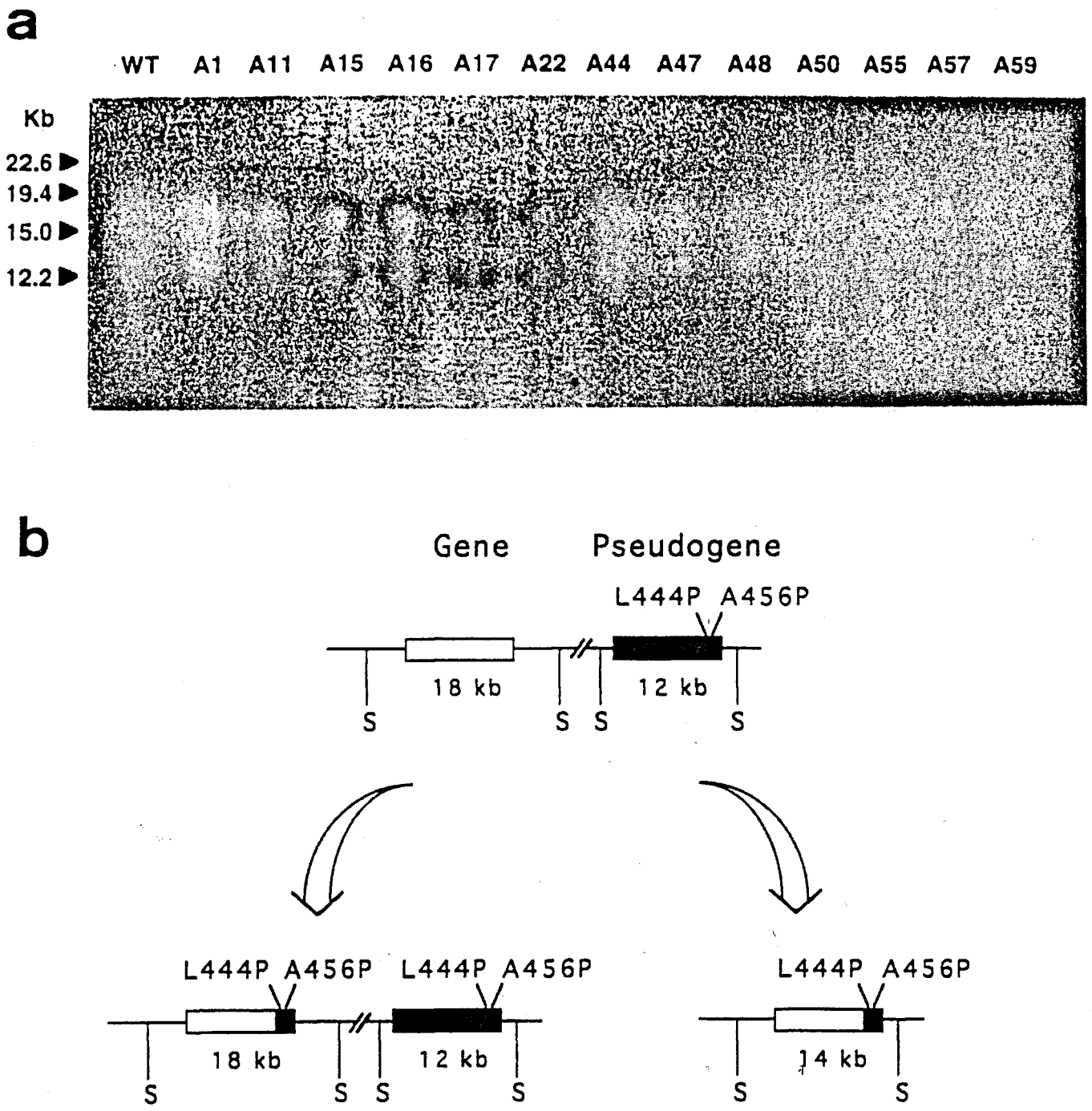

FIGURA 7. *a*) Anàlisi mitjançant transferència de Southern de mostres de DNA genòmic digerides amb *Ssp*I. El carril de l'esquerra (WT) correspon a un control normal amb dues bandes d'aproximadament 18 i 12 kb respectivament. Els pacients A17, A22, A50, A57 i A59 presenten el patró normal de dues bandes. Els pacients A1, A11, A15, A16, A44 (i el seu germà, A47) i A55 presenten una banda extra de 16 kb. En la mostra del pacient A48 es veuen les bandes de 18 i 12 kb més una banda addicional de 14 kb. *b*) Possibles models per als diferents al·lels RecNciI. Els que presenten el patró amb les bandes de 18 i 12 kb (esquerra) es podrien haver format per conversió gènica mentre que l'al·lel que presenta la banda de 14 kb (dreta) podria ser un gen de fusió resultant d'un entrecreuament desigual.

(Cormand *et al.*, 1998*a*). Així mateix, hem comprovat que l'haplotip conservat a l'entorn de la mutació N370S en els pacients espanyols és semblant al dels pacients jueus asquenasites (treball en preparació). No hi ha dades sobre els jueus sefardites, originaris de la península Ibèrica, però les nostres dades suggereixen que la mutació a Espa-

nya podria tenir l'origen en un cromosoma jueu. Si això es cert, els jueus sefardites portadors de la mutació N370S haurien de tenir el mateix haplotip, i per tant la mutació s'hauria originat abans de la separació entre les dues branques dels jueus.

L'anàlisi dels haplotips al voltant de les mutacions L444P (Cormand *et al.*, 1998*a*) i D409H (Chabás *et al.*, 1998) demostra que aquests canvis van tenir lloc repetidament, és a dir, que es tracta de mutacions recurrents. El fet que aquestes mutacions estiguin presents en el pseudogèn fa pensar en un procés reiterat de transferència de material genètic del pseudogèn cap al gen, potser per un mecanisme de conversió gènica.

AL·LELS REC, RECOMBINACIÓ O CONVERSIÓ GÈNICA?

S'han descrit alguns al·lels complexos que contenen diverses mutacions que estan normalment presents en el pseudogèn. Els més freqüents són l'al·lel RecNciI (amb les mutacions L444P i A456P i el canvi silenciós V460V, 1497G->C en l'àmbit nucleotídic) i l'al·lel RecTL (amb els mateixos canvis que RecNciI més la mutació D409H). Aquests al·lels podrien haver-se originat mitjançant un procés d'entrecreuament desigual entre gen i pseudogèn o per conversió gènica. En aquest darrer cas l'estructura genòmica al voltant del gen, que es pot analitzar utilitzant enzims de restricció i Southern, no variaria, mentre que un procés de recombinació alteraria el patró de bandes que s'obtenen mitjançant aquestes tècniques.

En general s'anomena «Rec» als mutants que contenen els canvis nucleotídics esmentats sense tenir en compte el mecanisme mitjançant el qual s'ha produït la mutació. En l'estudi dels pacients argentins (Cormand *et al.*, 1998*b*) hem identificat un elevat nombre d'al·lels RecNciI. En l'anàlisi per Southern d'aquests al·lels s'observen tres patrons diferents (figura 7a). Alguns dels pacients portadors de l'al·lel RecNciI presenten un patró similar al dels individus sans, compatible amb una hipòtesi de conversió gènica com a mecanisme generador de la mutació (figura 7b). D'altra banda, un dels pacients presenta una banda extra en el Southern que suggereix la presència d'un producte de fusió generat per un procés de recombinació entre gen i pseudogèn. Sorprenentment, alguns pacients presenten un tercer patró de bandes diferent dels anteriors no descrit fins ara. Actualment estem estudiant l'estructura molecular d'aquest al·lel mutant i el possible mecanisme que l'ha generat.

PERSPECTIVES FUTURES

Actualment estem posant a punt les tècniques d'expressió dels al·lels mutats. L'objectiu és caracteritzar la β-glucocerebrosidasa mutada i comparar-ne les propietats bioquímiques amb les de l'enzim normal. En el cas de mutacions noves, aquest estudi ens permetrà confirmar que el canvi trobat és realment la mutació causant de la malaltia. A més, conèixer les característiques de l'enzim pot resultar útil per a la prognosi de la malaltia. Per a l'expressió estem utilitzant d'una banda el sistema de baculovirus, caracteritzat per una gran eficiència de producció de proteïna heteròloga, i de l'altra, hem començat a utilitzar adenovirus com a vectors. Aquest darrer mètode utilitza cèl·lules humanes en cultiu i, en conseqüència, pot resultar interessant de cara a possibles aplicacions futures relacionades amb la teràpia gènica com a alternativa als protocols actuals, basats en sistemes retrovírics.

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