Comparison of RNase A, a chemical cleavage and GC-clamped denaturing gradient gel electrophoresis for the detection of mutations in exon 9 of the human acid β -glucosidase gene

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ABSTRACT

Gaucher disease (GD), which results from mutations in the human acid β -glucosidase (β -Glc) gene, was used as a model system to compare the utility of three methods capable of detecting single base substitutions. PCR-amplified β -Glc exon 9 sequences of GD patients were screened for single base mutations by GC-clamped denaturing gradient gel electrophoresis (DGGE) and RNase A cleavage of RNA-DNA heteroduplexes, and by chemical (hydroxylamine/osmium tetroxide) cleavage of dsDNA heteroduplexes. PCR products showing abnormal behaviour were cloned and sequenced. Three new point mutations were detected by this strategy. A G to C (Asp⁴⁰⁹ to His⁴⁰⁹) substitution was present in two Type 1 and one Type 3 GD patients; an A to T transversion (Asp⁴⁰⁹ to Val⁴⁰⁹) was detected in only a single Type 3 individual, and a G to T mutation (Val³⁹⁴ to Leu³⁹⁴) was present in one Type 1 and one Type 3 patient. GD thus exhibits extensive molecular heterogeneity, with at least five single base mutations in β -Glc exon 9. In every case verified by ASO hybridization, DGGE had correctly identified the presence of the three new mutations, as well as the two previously described exon 9 mutations. In comparison, although RNase A and the chemical method were both able to detect some of these mutations, neither method reproducibly detected all of them. Additionally, DGGE was the only method that was able to reliably determine whether a given mutation was present homozygously or heterozygously. These results suggest that GC-clamped DGGE may be a more reliable and informative screening method for point mutation detection.

INTRODUCTION

As the number of disease-related, cloned genes increases, there will be increasing interest in methods to detect mutations found in patient material. Direct sequencing of isolated mutant clones, or of genetic material amplified by the polymerase chain reaction (PCR; 1), will be necessary as a final step to identify the exact base changes. However, rapid screening methods also are needed to roughly localize mutations, and to identify groups of mutants before selecting candidates to sequence. Additionally, future data obtained from the human genome project may lead to an explosion in the number of genes that are found to be tightly linked to given diseases. Rapid screening for polymorphisms/mutations within such candidate disease-causing genes of patients and their families would also be useful in establishing a relationship between orphan genes and diseases.

Denaturing gradient gel electrophoresis (DGGE; 2) and RNase A cleavage (3) have been widely used to detect single base mutations. However, neither of these methods could detect all point mutations. In comparison, two recent reports suggested methods for detecting all possible single base mutations: the hydroxylamine/osmium tetroxide chemical cleavage method (4) and DGGE of PCR products which have been modified by GC-clamps (5). These methods have not been directly compared for their sensitivity, accuracy and

reproducibility in identifying single point mutations. For this purpose, Gaucher disease (GD) was chosen as a model system.

GD, the most prevalent lysosomal storage disease, results from inherited defects of acid β -glucosidase (β -Glc; EC 3.2.1.45, D-glucosyl-N-acylsphingosine glucosylase; 6). Clinical features including hepatosplenomegaly and skeletal abnormalities are present to varying extents, but the major types have been delineated by the absence (Type 1) or presence and severity (Types 2 and 3) of neuronopathic symptoms. Type 1 GD has the highest incidence within the Ashkenazi Jewish population, and Type 3 GD was first described in the Swedish population. However, all Types of GD are panethnic. Molecular studies indicate that GD results from single base substitutions in the β -Glc gene, and that heterogeneity of causative mutations exists both between and within the types (7, 8, 9, 10, 11). To date, four allelic point mutations have been described. Two mutations [a G to A transition in exon 5, Arg¹²⁰ to Gln¹²⁰ (9) and a C to G transversion in exon 9, Pro⁴¹⁵ to Arg⁴¹⁵ (10)] were found in single families (11). In comparison, an A to G transition in exon 9 [Asn³⁷⁰ to Ser³⁷⁰ (8)] occurred frequently but only in Type 1 GD patients of varying ethnicity (8, 11). Also, a T to C transition in exon 10 (Leu⁴⁴⁴ to Pro⁴⁴⁴) was present at high frequency in Type 2 and 3 GD patients, and occurred heterozygously at low frequency in Type 1 GD patients (7, 10, 11). Extensive molecular analyses of a large GD population indicated that the genotype could be determined completely by the described mutations in only about 30% of patients. The varying clinical phenotypes and ethnic origins of these incompletely characterized patients indicated the existence of multiple additional causal mutations in the β -Glc gene (11).

To further investigate the molecular heterogeneity of this model disease, β -Glc exon 9 specific sequences of incompletely characterized patients were amplified by PCR, and screened for the presence of single point mutations by RNase A, chemical cleavage and DGGE. Exon 9 was selected for analysis because two of the four previously characterized mutations were located in this exon, suggesting that it may code for an important structural or catalytic domain. Three new mutations in this exon were detected and their frequencies in the GD population were determined. The results of this screening provided for direct comparisons of the efficiency of three different methods for the detection of both known and unknown point mutations. DGGE of RNA-DNA heteroduplexes containing GC-rich clamps was found to be more reliable at mutation detection than either RNase A analysis (3) of RNA-DNA heteroduplexes or chemical cleavage (4) of ds DNA heteroduplexes.

MATERIALS AND METHODS

Materials

The following reagents were obtained from commercial suppliers and used under the recommended conditions: restriction endonucleases, DNA polymerase I large fragment (Klenow), T4 polynucleotide kinase, *Thermus aquaticus* (Taq) polymerase (New England Biolabs, Beverly, MA); Taq polymerase (Perkin Elmer Cetus, Norwalk, CT); S1 nuclease (Sigma, St. Louis, MO); RNase A (Worthington, Freehold, NJ.); radiolabelled nucleotides (New England Nuclear, Boston, MA); unlabelled nucleotides (Fisher, Springfield, NJ); hydroxylamine, osmium tetroxide, piperidine, pyridine, diethylamine (Aldrich Chemical Co., Milwaukee, WIS).

Patient description

Genomic DNA from normal individuals and GD patients were extracted from fibroblast or lymphoblastoid cell lines as described (11). The patient population has been described

in detail (11). All patient samples were obtained by informed consent, or assent of minors, according to institutional and NIH guidelines.

Preparation of probes and primers

Oligonucleotides were synthesized on an Applied Biosystems 380B DNA Synthesizer. β -Glc specific RNA probes for RNase A cleavage analysis were transcribed from plasmid pIBI30-2257 that contained a β -Glc genomic insert between the T7 and T3 RNA polymerase promoters [β -Glc bases #4729 to 6988; numbering throughout this manuscript is according to ref.12; EMBL/Genbank Data Libraries Accession No. J03059]. pIBI30-2257 was obtained by subcloning into the vector pIBI30 (IBI, New Haven, CT) the relevant SmaI/SacI fragment from a β -Glc genomic clone (N3; 11). Transcription reactions were carried out in the presence of [α - 32 P]CTP as described (13). To produce a sense probe, the pIBI30-2257 was digested with EcoRI, and transcribed with T3 polymerase. For the antisense probe, pIBI30-2257 was digested with XbaI, and transcribed with T7 polymerase.

DNA probes for the chemical cleavage method were produced from pIBI30-2257 (sense probe) or from pIBI31-2257 (antisense probe). pIBI31-2257 was constructed in the same way as pIBI30-2257, but contained the β -Glc insert in the opposite orientation. Single stranded copies of these constructs were obtained by superinfection of plasmid-containing bacteria with a helper phage, as described by the supplier (IBI). 50 pmol of the antisense strand primer XBA3EX9 (5'-CCTCTAGAAA TGGGGGTGCC CGCCCTCCA-3', a 29-mer oligonucleotide including a 5' XbaI site and 21 bases complementary to β -Glc sequences #6009–6029) or the sense strand primer ECOR5EX9 (5'-GGGAATTCGT GTTGAACCTT TGTCTCTT-3', a 28-mer containing an EcoRI site and 20 bases complementary to β -Glc sequences #5800–5819), were annealed to 0.6 pmol of the single-stranded construct in 10 μ l of 75 mM NaCl, 15 mM Tris-HCl pH7.5, 15 mM DTT, 15 mM MgCl₂, by boiling for 3 min followed by incubation at room temperature for 30 min. The primer was then extended for 30 min at room temperature in 30 μ l of the above buffer that contained the following additional reagents: 17 pmol each of [α - 32 P]dATP and [α - 32 P]dCTP, 17 μ M each of dGTP and dTTP, 2.5 μ M each of dATP and dCTP, and 5 units of Klenow polymerase. The reaction was stopped by addition of 50 μ l 10mM EDTA, followed by phenol-chloroform (50:50, v/v) extraction and ethanol precipitation. The sample was then digested with either 16 units of AluI to produce an antisense probe complementary to β -Glc bases #5752 to 6029, or with 16 units of StuI to produce a sense probe complementary to β -Glc bases #5800 to 6039. The probes were purified by electrophoresis through a 6.5% polyacrylamide gel containing 7 M urea and, following autoradiography, the band of size corresponding to the probe was cut out and eluted.

For DGGE, an antisense probe containing 40 nt of GC-rich sequence at one end was transcribed (13) by T7 polymerase from the EcoRI digested plasmid pIBI31-CLEX9. This construct contains β -Glc sequences #5800–6039 obtained from PCR-amplification of the exon 9 region of genomic clone N3 (11). The sense primer used was ECOR5EX9 (see above) and the antisense primer was XBACL3EX9 (5'-GGTCTAGACC CCGCCCCGC CCGCCGCGCC CCGCGAGCCC CCGCCCGCCC TGGTATGGAA TGGGGGTG-3', a 68-mer containing an XbaI site, a 40 base GC-rich sequence, and 20 bases complementary to β -Glc sequences #6020–6039). The PCR product then was digested with the restriction enzymes EcoRI and XbaI, and ligated into EcoRI/XbaI digested pIBI31.

Mutation notation

Previously, mutations were numbered according to the amino acid substitution that resulted and/or according to the position of the base change in the cDNA, where the A of the most

5' AUG was designated base #1 (for example, ref. 9). A potential problem with this nomenclature is that any mutations discovered in non-coding regions cannot be numbered using this system. Since the entire β -Glc gene has recently been sequenced (12), it is now possible to assign numbers to all point mutations. Thus, in this paper, mutations were named according to the number of the genomic nucleotide which was altered and the resultant base difference: 5841G (8); 5912T, 5957C, 5958T (this study); 5976G (10); 6433C (7).

PCR conditions

For exon 9 screening, PCR was used to amplify β -Glc specific genomic DNA sequences using intron-specific primers whose 3' ends terminate between 5 and 20 nucleotides from the exon 9 boundaries. Patient genomic DNA (1 μ g) or plasmid DNA (50ng) was added with 50 pmol of each amplimer (ECOR5EX9 and XBA3EX9 for the RNase A and chemical cleavage methods, or amplimers ECOR5EX9 and XCL3EX9 for DGGE) in 100 μ l of PCR buffer. The buffer used was as described by New England Biolabs (Beverly, MA), or from a Geneamp™ kit (Perkin-Elmer Cetus, Norwalk, CT). Denaturation was at 94°C for 7 mins, after which 2.5 units of Taq polymerase were added. The mixtures were overlaid with 100 μ l of mineral oil (Sigma), and 30 cycles of amplification (30 sec at 94°C followed by 5 min at 66°C) were carried out (DNA Thermal Cycler, Perkin Elmer-Cetus).

For dot blot analyses, selective amplification of exon 9 structural gene-derived sequences was accomplished as described (11). The sense amplimer was ECORDELEX9 (5'-CCGAATTCTG AACCCCGAAG GAGGACC-3', a 27-mer containing an EcoRI site and 20 bases complementary to bases # 5885–5904, which are deleted in the pseudogene) and the antisense amplimer was XBA3EX9 (see above).

RNase A Cleavage

RNase A cleavage was performed as described (3). Briefly, the labelled RNA probe was hybridized to denatured exon 9 PCR products and incubated in 10 mM Tris-HCl pH7.5, 1 mM EDTA, 200 mM NaCl and 100 mM LiCl containing RNase A at 5 μ g/ml, 25 μ g/ml or 80 μ g/ml for 1 hr at 25°C. The reaction was stopped by addition of SDS to 0.5% and proteinase K to 0.25 mg/ml followed by incubation for 15 min at 37°C. The samples then were extracted with phenol:chloroform (50:50 v/v), ethanol precipitated, resuspended in loading mix (14) boiled for 2 mins, and analyzed by electrophoresis in an 8% polyacrylamide gel containing 7 M urea. The conditions for RNase A cleavage were optimized using three plasmids obtained from Dr. R. Myers that contained β -globin gene inserts: two plasmids, π svwt2 and π sv β °39, contained a normal and a mutant gene, respectively, and one plasmid was an RNA transcription vector that contained a normal β -globin gene (pSP6105; ref.3).

Chemical cleavage

Heteroduplex formation, hydroxylamine and osmium tetroxide modification, piperidine cleavage and analysis on gels was as described by (4) with the following modifications. A 10–75 molar excess of unlabelled PCR product over labelled DNA probe was used for heteroduplex formation and about 18,000 cpm (range 6000–30,000 cpm) of the hybridization mix was distributed to each assay tube. Chemical reactions were performed in 2 μ g sonicated salmon sperm DNA and heteroduplexes were reacted with 3.8 M (final concentration) hydroxylamine for 120 min at 37°C, or with 0.5% (final concentration) osmium tetroxide for 10 min at 37°C. Cleavage products were analyzed on 8% polyacrylamide gels containing 7 M urea.

Denaturing gradient gel electrophoresis (DGGE)

β -Glc exon 9 sequences were amplified using one primer that contained a GC clamp

(XCL3EX9, see above), hybridized with a complementary RNA probe, and treated with S1 nuclease to remove unreacted probe as described previously (15). The resulting heteroduplexes then were analyzed by polyacrylamide gel electrophoresis (PAGE; 6.5%), the gel was autoradiographed, and the band corresponding to the structural gene was cut out and eluted. The gel purified sample was ethanol precipitated, and resuspended in water. Approximately 3,000 cpm per lane was subjected to PAGE (6.5%) in a linearly increasing gradient of denaturant (100% denaturant = 7 M urea/60% (v/v) formamide). The design of the electrophoresis apparatus was as described (16) and the running buffer (40mM Tris acetate, 1 mM EDTA) was maintained at 55°C.

Dot blot analysis by allele specific oligonucleotide (ASO) hybridization

ASO hybridization procedures were as described (11). The ASOs used to detect the mutation 5957C were 5'-AACGTGTCCTTGGTG-3' (normal) and 5'-AACGTGTGCTTGGTG-3' (mutant), using washing temperatures of 46°C and 52°C, respectively, in 4×SSC. The ASOs used to detect the mutation 5958T were 5'-ACCAAGGACACGTTT-3' (normal) and 5'-ACCAAGGTCACGTTT-3' (mutant), using washing temperatures of 47°C. The ASOs used to detect the mutation 5812T were 5'-TTACGCACCCAATTG-3' (normal) and 5'-TTACGCAACCAATTG-3' (mutant), using washing temperatures of 44°C and 42°C, respectively.

RESULTS

RNase A Cleavage

RNA probes of normal sequence were hybridized to the products of PCR amplification of β -Glc exon 9, and the resulting RNA-DNA duplexes were treated with RNase A, which preferentially cleaves the RNA strand at mismatched bases (3). Therefore, treatment of a hybrid formed with normal sequences ideally results only in trimming of excess unhybridized probe and on electrophoresis produces only a single band equal to the length of the PCR product. In contrast, the presence of mutant sequences produces smaller molecular weight bands due to cleavage at mismatches. The conditions for RNase A cleavage were optimized using plasmids obtained from Dr. R. Myers. Mismatched RNA-DNA hybrids produced by using these plasmids were easily cleaved at low concentrations of RNase A (5 μ g/ml; Fig.1). However, it has been reported that some mismatches require longer incubation times or higher concentrations of RNase A (3). Therefore, all patient samples were treated with three different concentrations of RNase A (5, 25, and 80 μ g/ml). Mutations detected generally were seen at all concentrations, although the 80 μ g/ml resulted in very high background (data not shown).

Fig.1 shows examples of results obtained for GD DNAs at the two lowest enzyme concentrations in two different experiments, one using the antisense probe (DNAs #61 and #63), one using the sense probe (DNAs #41, #42, and #52). Multiple background bands which were characteristic for each probe were obtained at all RNase A concentrations for all GD DNAs. Typical background patterns for these probes are shown by DNAs #42 and #63. However, samples #41 and #61 clearly exhibit a new set of bands approximately 158 bp in length, indicating the presence of a new mutation in exon 9 in at least one allele of each of these patients. In both samples, a second lower band about 72 bp in length would also be expected, but was not observed. Patient #41 is known to have an allele with the exon 9 Type 1 mutation 5841G (11), but on comparison with DNAs known to be negative for this mutation, no specific cleavage bands of the sizes predicted for this mutation (41 and 189 bp) were distinguishable on RNase A cleavage of this DNA (Fig.1), or of other GD DNAs known to contain at least one copy of this mutation (data not shown).

Figure 1. Screening for mutations by RNase A cleavage of RNA-DNA duplexes. RNA probes were hybridized to DNA from either control plasmids or PCR-amplified exon 9 sequences of the β -Glc gene of a group of GD patients. Duplexes then were incubated with RNase A and electrophoresed on 8% polyacrylamide gels containing 7M urea. The positions of cleaved bands in the mutant plasmid and in GD DNAs #41, #52 and #61 are indicated by arrows.

Patient #52 was observed to have a unique pattern of cleavage, yielding a cluster of bands approximately 113 bp in length, consistent with cleavage approximately in the center of the RNA probe.

Experiments using hybrids that had been S1 nuclease treated and gel-purified also were performed in an attempt to lower the background observed with RNase A cleavage, but this did not give improved results (data not shown).

Chemical Cleavage.

DNA probes were used to form hybrids with the unlabelled PCR products. Subsequent reaction with hydroxylamine should specifically modify unpaired C residues, while osmium

Figure 2. Screening for mutations by chemical cleavage of ds DNA duplexes. DNA probes were hybridized to PCR-amplified exon 9 sequences of the β -Glc gene of a group of GD patients. Duplexes then treated with hydroxylamine and piperidine, and electrophoresed on 8% polyacrylamide gels containing 7M urea. The positions of cleaved bands in patients #41 and #52 are indicated by arrows. Lengths of cleaved bands were estimated by comparison with a sequencing reaction which was run on every gel (not shown). C, control hybrids incubated with water before piperidine treatment; H, test hybrids incubated with hydroxylamine before piperidine treatment.

tetroxide should modify unpaired T residues (4). The duplexes then were cleaved at modified residues by incubation with piperidine. Results of chemical cleavage analysis for a group of GD patients are shown in Fig. 2. In this experiment, PCR amplified β -Glc exon 9 was hybridized with an antisense probe and treated with hydroxylamine. A 'background' ladder of cleavage was observed even with perfectly matched duplexes made from DNA known to contain only normal exon 9 sequences (data not shown). However, superimposed on this background, additional patient-specific cleavage bands approximately 158 bp in length can be seen for patient #41. The second lower band about 80 bp in length predicted from this cleavage was not observed. These results are consistent with those obtained by RNase A, and predicted replacement of a C residue in the antisense strand (a G residue in the sense strand) with another base on a mutant allele in patient #41. In comparison, hydroxylamine treatment of heteroduplexes formed using DNA from patient #61 failed to produce any specific cleavage bands (data not shown). This result suggested that the

A. MELTING MAPS OF EXON 9 (WITH AND WITHOUT GC-CLAMP)

B. PERPENDICULAR DENATURING GRADIENT GEL ELECTROPHORESIS

exon 9 mutation in this patient detected by RNase A cleavage did not result from substitution of a C residue in the antisense strand. Additionally, patient #52 DNA produced an approximately 113 bp cleavage band, which was similar to the result obtained with RNase A cleavage.

Similar experiments were conducted with osmium tetroxide, but high background cleavage was obtained with three different batches of osmium tetroxide and over a wide range of osmium tetroxide and piperidine concentrations. While it was possible to detect cleavage of T-mismatches produced using two different sequenced plasmid DNAs, the method consistently failed to detect a T-mismatch using a third plasmid DNA. Also, osmium tetroxide treatment of genomic DNA led to high background cleavage, obscuring the detection of any mutations (data not shown).

Denaturing gradient gel electrophoresis (DGGE).

The effect of the addition of a 40 bp GC-clamp on the expected melting progression along a ds DNA PCR product was calculated as described (2; Fig. 3). The β -Glc sequences spanned positions #5781 to #6020, and contained all of exon 9 as well as some flanking bases. For the molecule lacking a GC-clamp, strand dissociation was probable at a temperature lower than that required for melting of the coding region. Therefore, for this fragment there was no temperature at which a partially melted molecule was favored by the equilibrium, and thus it could not be examined for base substitutions. In contrast, the addition of a GC-clamp raised the temperature at which strand dissociation was probable to significantly above that at which the coding region melted, thereby converting this molecule into two distinct domains. Consequently, the entire coding region became a low melting domain (LMD), which was amenable to analysis by DGGE. Previous analyses indicated that the same relative order of domains found in ds DNA molecules was also found in RNA-DNA molecules (15), suggesting that a GC-clamp would also convert an RNA-DNA duplex into two melting domains.

To obtain a GC-clamp at one end of the PCR product, PCRs were performed using one primer that contains 40 bases of a GC-rich sequence, in addition to the 20 nucleotides complementary to the β -Glc gene. These products then were hybridized to RNA probes that also contained these extra sequences. Analysis of these heteroduplexes by DGGE distinguishes those containing single base mismatches in their LMDs by their altered melting behaviour. Mismatched duplexes are less stable than perfectly paired duplexes, and undergo partial melting, and thus retardation, at a lower concentration of denaturant than do perfectly matched duplexes.

Electrophoresis on a perpendicular denaturing gradient gel of RNA-DNA homoduplexes, formed by hybridizing PCR products either with or without a GC-clamp to a radioactively

Figure 3. Theoretical and experimental analyses of the melting behaviour of homoduplexes. Panel A. Theoretical melting maps of PCR-amplified β Glc exon 9 and its flanking regions. The abscissa represents positions along the PCR product, and the ordinate shows the temperature at which each base has an equal probability of helix or random chain configuration. The position of the coding region is indicated in the box diagram below the maps. Melting maps of the PCR product either with (continuous lines) or without (hatched lines) a 40bp GC-clamp at the 3'-end are shown. The temperature at which the dissociation constant for complete strand dissociation of the PCR product is 10^{-7} M is indicated by either a hatched line (unclamped) or a continuous line (GC-clamped). Panel B. Experimental melting patterns of homoduplexes after perpendicular DGGE. The mobilities of RNA-DNA homoduplexes (either with or without a 40bp GC-clamp) were examined on a denaturing 10–90% gradient perpendicular to the electric field. The samples were applied uniformly across the top of the gel. An arrow indicates the midpoint of the transitions from duplexes to single stranded molecules.

PARALLEL DENATURING GRADIENT GEL ELECTROPHORESIS

Figure 4. Identification of single base mismatches in RNA-DNA hybrids by electrophoresis in 6.5% polyacrylamide gels containing a denaturing gradient. The gradient (30–60%) was parallel to the electric field. The patterns of migration shown were obtained in two different experiments (panels A and B), when the homoduplex controls reached the approximate concentrations of denaturant indicated. Note that the relative migration of the mismatched hybrids changes with the percent denaturant.

labelled RNA probe, identified the concentration of denaturant at which their LMDs melt. Fig.3B shows that the presence of the clamp does not alter the concentration of denaturant at which the LMD melts. However, based on the theoretical melting maps, the clamp should prevent the molecule from undergoing strand dissociation at this concentration. Experiments using parallel denaturing gradient gels determined the electrophoretic conditions under which the LMDs of homoduplexes melt in the denaturing gradients (data not shown).

The same samples that had been analyzed by RNase A and chemical cleavage were examined by DGGE (Fig.4). DGGE in the absence of a GC-clamp failed to detect any mutations (data not shown). However, GC-clamped DGGE detected all previously characterized mutations. Fig.4 shows a single retarded band for patient #27, who was homozygous for the exon 9 mutation 5841G. Two bands (one normal, one mutant) were observed for patient #55, who was demonstrated to be heterozygous for the mutation 5976G (10). These results show that DGGE can clearly distinguish heterozygotes from homozygotes for these alleles.

It should be noted that the highest melting domain (HMD) of the GC-clamped hybrid melted at concentrations of denaturant only slightly higher than those required to melt the LMD. Therefore, we were confident that the hybrids had reached the correct denaturant concentration to melt the LMD only if two time points were analyzed on each gel: one which gave sharp bands due to retardation after melting of the LMD but before strand dissociation, and a slightly longer time point that exhibited blurry bands (resulting from HMD strand dissociation), verifying that the normal controls had passed through the concentration of denaturant sufficient to melt the HMD. Mutations that cause only a small change in stability of the heteroduplex would be detectable only during a very small window of denaturant concentration. For example, the mutation in patient #55 was clearly visible over multiple time points, whereas that in patient #27 was visible only shortly before strand dissociation (Fig. 4). A further reason for examining the migration of bands at different time points is that the relative migration of retarded species may change (Fig. 4), increasing the likelihood of distinguishing between different mutations.

Analysis of GD DNA by GC-clamped DGGE also detected all mutations tentatively identified by RNase A or chemical cleavage screening. Fig. 4 shows that PCR products derived from patient #61 showed two bands on DGGE: one with identical mobility to the normal control indicating the presence of a normal sequence in exon 9 on one allele, and one whose mobility was retarded indicating the presence of a mutation in exon 9 on the other allele. This mutation is labelled 5958T in Fig. 4. DNA from patient #41 showed two different retarded bands: one with a mobility identical to that seen with DNA which was homozygous for mutation 5841G, and one with a mobility not previously seen in any DNA, suggesting the presence of a new mutation. This mutation is labelled 5957C in Fig. 4. It should be noted that the indicative bands in patients #41 and #61 were retarded to different degrees, suggesting the presence of different mutations in these two patients. Similar analyses of DNA from patient #52 also identified two retarded bands: one consistent with mutation 5841G, and a second with a new migration pattern. This latter finding suggested the presence of a new mutation (labelled 5912T in Fig. 4).

DGGE screening also indicated the presence of retarded bands in the PCR products derived from patients #44 and #74 (Fig. 4). Two of these bands had mobilities similar to that of the most retarded band in patient #41 (mutation 5957C). It should be noted that the signal obtained from the mutant band in patient #44 was consistently 5-to-10-fold lower than that obtained with the normally migrating band (Fig. 4). This probably reflects inefficient PCR amplification of this allele. A second differently retarded band was seen in patient #74, indicating that both alleles in this patient contained mutations in exon 9. This second band had similar mobility to that of the most retarded band seen in patient #52 (mutation 5912T; Fig. 4).

Characterization of new exon 9 mutations

The PCR products of exon 9 amplification of DNAs from patients #41, #52, #61 and #74 were cloned directly into pIBI31, using the EcoRI and XbaI sites that had been

A. SEQUENCING OF CLONED PCR PRODUCTS

B. ASO HYBRIDIZATION

Figure 5. Characterization of mutations. Panel A. PCR products behaving abnormally in the screening procedures were cloned and sequenced. The relevant portions of the sequencing gels are shown for the three newly identified mutations. Panel B. Selective hybridization of oligonucleotide probes to PCR-amplified genomic DNA. Oligonucleotides complementary to either the normal or mutant sequence of the newly identified mutations were used to screen a population of GD patients. Plasmid controls were either the normal genomic clone N3, or the cloned PCR-products in which the mutation was first identified. Results obtained for a different group of six DNAs is shown for each probe.

incorporated into the amplimers. Additionally, the retarded bands seen for patients # 52 and # 74 on DGGE were cut out, and the β -Glc DNA was PCR amplified before cloning. Several clones obtained from both procedures were sequenced (17; Fig. 5A). Clones derived from patient # 41 revealed two different sequences: one allele contained the exon 9 mutation 5841G, while the other allele contained a G to C substitution at base # 5957 that predicted a substitution of Asp⁴⁰⁹ with His⁴⁰⁹ (Fig. 5A). The involvement of a G residue is consistent

Table 1. Characterization of patients in which new mutations were identified.

Patient	Type	Ethnic Origin	Mutations				
			Exon 9				Exon10
			5912T	5957C	5958T	5841G	6433C
#52	Type 1	Ashkenazi Jewish	+			+	
#41	Type 1	Greek		+		+	
#44	Type 1	Racially mixed S.African		+			+
#61	Type 3	Caucasian			+		+
#74	Type 3	Ashkenazi Jewish / Irish	+	+			+

Both new mutations and previously reported mutations (11) are shown. + indicates the presence of the mutant sequence.

with the detection of this mutation by the chemical method, while its position 158 bp from one end of the PCR-amplified region is consistent with the size of the cleavage bands seen by both RNase A and chemical cleavage. Since this substitution also was present in the exon 9 homologous region of the β -Glc pseudogene (12), this second 'allele' could have arisen from strand jumping during the PCR. To determine whether this sequence was present in the β -Glc structural gene, PCR amplification of genomic DNA from patient #41 was performed using primers which amplify only structural gene sequences (11). Dot blot analysis of these PCR products using ASO hybridization revealed that mutation 5957C was present, heterozygously, in the structural gene (Fig. 5B).

Sequencing of clones derived from patient #61 revealed one allele that was identical to the normal sequence throughout exon 9, and a second allele that contained an A to T transversion at position #5958 (Fig. 5A) that predicted an Asp⁴⁰⁹ to Val⁴⁰⁹ substitution. Sequencing of clones derived from patient #52 verified the presence of the mutation 5841G, as expected from previous screenings for known mutations (11). The other allele of patient #52 contained a G to T change at position #5912 (Fig. 5A), that predicted a Val³⁹⁴ to Leu³⁹⁴ substitution. The nature and location of this mutation (113 bp from one end of the PCR-amplified region) were consistent with the results obtained by RNase A and chemical cleavage. Sequencing of clones derived from patient #74 revealed one allele that contained the mutation 5957C, while the other allele contained the mutation 5912T. These results were also consistent with migration patterns seen on DGGE, and all these mutations were verified to be present, heterozygously, in genomic DNA by ASO hybridization of pseudogene-free PCR products, as described above (Fig. 5B).

Frequency of the new mutations in the GD population.

A population of 44 GD patients of varying clinical phenotype and ethnicity was then screened for the presence of the sequenced new mutations in their structural genes by pseudogene-free PCR amplification of exon 9 followed by ASO hybridization (11). This population was the same as that analyzed for unknown mutations by RNase A, chemical cleavage, and DGGE. Therefore the screening by ASO hybridization functioned as a check for false

negatives that may have been obtained by the other methods. The results are presented in Table 1. Mutation 5912T was present heterozygously in patients #52 and #74, and mutation 5957C was present heterozygously in patients #41, #44, and #74. Mutation 5958T was unique to and heterozygous in patient #61. These results were as expected on the basis of the DGGE observations.

DISCUSSION

In the present study, three methods capable of detecting single base substitutions— RNase A cleavage, chemical cleavage, and GC-clamped DGGE—were compared for their utility in detecting mutations in exon 9 of the β -Glc gene. All three methods were able to detect mutations, as exemplified by results obtained with patients #41 and #52. RNase A cleavage was the quickest and easiest method, but it has been reported to be capable of detecting only approximately 30% of mismatches in each probe (3). In this study, it was able to detect only three of the five exon 9 mutations examined (all three of the new mutations, but neither of the two previously reported mutations: 8, 10). Additionally, the background cleavage of perfectly matched bases made the detection of partially cleaved mutations and poorly amplified alleles difficult, and may account for the fact that mutation 5957C was detected consistently in only one of the three patients in which it was present (#41), and mutation 5912T in only one of two patients in which it was present (#52).

Similar problems were encountered with the chemical cleavage method. Using plasmid DNA and probes of both sense, this method has been reported to detect all base substitutions, albeit with varying efficiencies of cleavage (4). Hydroxylamine treatment was able to detect mutations resulting in a C mismatch in the probe (i.e. the antisense C to G transversion at position #5957 in patient #41, and the antisense C to A change at position #5912 in patient #52). However, the resultant high background produced on osmium tetroxide treatment made it impossible to distinguish T mismatches in GD DNAs. Since this problem has not been reported previously, it may be specific for the batches of osmium tetroxide used in this study. However, it was observed using three different batches.

In contrast to the above methods, DGGE is not dependent on the preferential cleavage of mismatched bases over perfectly matched bases, and consequently does not have inherent background cleavage. DGGE can separate mutants on the basis of sequence-dependent melting properties of double-stranded nucleic acid molecules by passage through a denaturing gradient gel (2). The addition of a GC-clamp to one end of the molecule (18) enables all domains to be examined, and amplification of genomic sequences by the PCR allows GC-clamps to be attached to all molecules (5). To further increase the sensitivity of DGGE, we have modified the method of Sheffield et.al.(5) by hybridizing the GC-clamped PCR products with RNA probes. Although GC-clamped homoduplexes that differ in sequence by a single base pair usually are separable by DGGE (5), heteroduplexes containing mismatches are less stable than homoduplexes (2), resulting in increased resolution and the ability to detect all mutations. Additionally, the sensitivity of the assay was increased by using radiolabelled heteroduplexes, thereby decreasing the amount of PCR product needed for each analysis. Under these conditions, DGGE was able to detect both known exon 9 mutations, as well as identify the three new ones. Neither the RNase A nor the chemical cleavage methods provided similar accuracy. Moreover, DGGE definitively identified heterozygotes for all alleles, and distinguished two different mutations (5957C and 5958T) which produced similar sized cleavage bands with RNase A digestion. The low background of DGGE and its ability to separate alleles also allowed for the detection

of unequally amplified alleles: the mutant band in #44 consistently exhibited a 5-to-10-fold lower signal than the normal band on DGGE (e.g. Fig. 4). Results in Fig. 4 also emphasize the need to monitor the behaviour of the hybrids during DGGE at different positions in the gradient to facilitate distinguishing alleles.

Three previously unreported mutations in the β -Glc gene have been identified in the present work. All three mutations were present in Type 3 GD patients, but two (5912T and 5957C) also were present in Type 1 patients. These results suggest that combinations of alleles may modulate the severity of the phenotype. However, the results presented here cannot determine whether these mutations are disease-causing mutations: the characterization of the products expressed from each mutant allele will be required. Nevertheless, it is interesting that the only changes found resulted in amino-acid substitutions. Mutation 5957C was present in three different GD patients (one Ashkenazi Jewish/Irish Type 3, one Greek Type 1, and one South African Type 1), and predicts a substitution of an acidic amino acid (Asp⁴⁰⁹) in the wild type for a basic amino acid (His⁴⁰⁹) in the mutant. This substitution therefore is likely to have a significant effect on the protein's secondary structure. Interestingly, the presence of this new exon 9 mutation in three individuals of widely varying ethnic backgrounds (Table 1) and its presence in the pseudogene (12) may indicate three independent mutational events due to gene conversion.

An additional new mutation, an A to T tranversion at position 5958 (Asp⁴⁰⁹ to Val⁴⁰⁹), was found to be present in a single individual with Type 3 GD. This change substitutes a charged acidic residue in the normal protein with an uncharged hydrophobic residue in the mutant, and thus suggests a substantial effect on protein secondary structure. Interestingly, this mutation affects the same codon as mutation 5957C, suggesting a functionally significant role for the encoded amino acid, and supporting a causative role for these two mutations in the production of GD. A third new mutation, 5912T, was present in two patients: one Ashkenazi Jewish Type 1 patient, and one Ashkenazi Jewish/Irish Type 3 patient. This substitutes a hydrophobic amino acid (Val³⁹⁴) for another (Leu³⁹⁴) of similar structure and properties. This substitution would not suggest a large effect on the protein structure and may represent a rare normal variant, rather than a disease-causing mutation.

It is interesting to note that three different mutations have been detected in patient #74: two in exon 9 on separate alleles (this study) and one in exon 10 (11; Table 1). It is not known which of the exon 9 mutations is coupled with the exon 10 mutation. However, two of these mutations (5957C in exon 9, 6433C in exon 10) are also found in the pseudogene, suggesting that one allele could have arisen by a gene conversion event involving at least two exons. cDNA isolation and sequencing will be required to confirm this speculation. Nevertheless, the presence of at least two different point mutations on one allele in patient #74 indicates the existence of complex alleles within the GD population. Such complex alleles have been previously postulated to explain the observation that both Type 2 and Type 3 GD patients have been characterized as homozygous for the exon 10 mutation (7, 11).

In conclusion, three new mutations within exon 9 of the β -Glc gene have been identified that are present at low frequency in the GD population. Thus a total of 5 mutations in exon 9 of the β -Glc gene have now been reported. Although the role of these three new mutations in the etiology of GD has yet to be demonstrated, these studies suggest an increasingly complex picture of the molecular heterogeneity of GD.

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