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A novel process for mutation detection using uracil DNA-glycosylase

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ABSTRACT

A novel process is presented for the detection of known mutations and polymorphisms in DNA. This process, termed glycosylase mediated polymorphism detection (GMPD) involves amplification of the target DNA using three normal dNTPs and a fourth modified dNTP, whose base is a substrate for a specific DNA-glycosylase once incorporated into the DNA. The work described here utilises uracil DNA-glycosylase as the specific glycosylase and dUTP as the modified dNTP. Primers are designed so that during extension, the position of the first uracil incorporated into the extended primers differs depending on whether a mutation is present or absent. Subsequent glycosylase excision of the uracil residues followed by cleavage of the apyrimidinic sites allows detection of the mutation in the amplified fragment as a fragment length polymorphism. Variation in the sizes of the fragment length polymorphisms generated, can be readily achieved through the use of inosine bases in place of adenine bases in the upper and/or lower primers. The GMPD process is also adaptable to solid phase analysis. The use of the process for detection of mutations in the *RYR1* and *CFTR* genes is demonstrated. Overall, the simplicity, specificity, versatility and flexibility of the GMPD process make it an attractive candidate for both small and large scale application in mutation detection and genome analysis.

INTRODUCTION

Detection of multiple different mutations and polymorphisms at specific locations in DNA is of prime importance for genetic diagnosis, molecular genotyping, molecular epidemiology and rapid identification of various traits in eukaryotic and prokaryotic organisms. The surge in genome research over the last decade and particularly since the advent of PCR has identified numerous mutations of significance for human and animal health and numerous mutations of commercial value (1). Mutation detection technology has progressed with advances in genome research and a variety of methods have been described for detection of known mutations. However, many of these methods suffer from drawbacks that limit their utility and there is considerable interest in the development of improved detection systems. An optimum system for mutation detection needs to offer specificity, sensitivity, flexibility, versatility and ease of use. In addition an optimum system should also be robust, allow rapid and high throughput of samples, be amenable to automation and be cost effective.

Here, we describe a novel process for mutation detection referred to as glycosylase mediated polymorphism detection (GMPD) which exploits the use of highly specific DNA-glycosylase enzymes to excise substrate bases incorporated into amplified DNA.

In this work, the DNA-glycosylase used is uracil DNA-glycosylase (UDG). UDG is a highly specific DNA repair enzyme which cleaves the N-glycosidic bond between the base uracil and the sugar deoxyribose in a DNA molecule and generates an apyrimidinic (AP) site. The AP site can subsequently be cleaved chemically or enzymatically (2,3). The enzyme recognises and releases uracil from both single stranded and double stranded DNA efficiently (4,5). Uracil can be generated readily in DNA through the incorporation of deoxyuridine triphosphate (dUTP) or through chemical or heat induced deamination of cytosine residues in DNA (6–8).

Here, we demonstrate the use of the GMPD process for accurate and specific detection of several point mutations in the *RYR1* gene causal of the inherited disorder malignant hyperthermia and the ΔF508 deletion mutation in the *CFTR* gene causal of cystic fibrosis.

MATERIALS AND METHODS

Oligonucleotide labelling and DNA amplification and dUTP incorporation

Human genomic DNA was prepared from blood and muscle samples as described previously (10). Primers (gel purified) (60 pmol) were end labeled by incubation with T4 polynucleotide kinase (New England Biolabs) in appropriate buffer and 50 µCi [γ-^{32}P]ATP (3000 Ci/mmol) (New England Nuclear) for 30 min at 37°C followed by ethanol precipitation to remove unused labelled nucleotide. Using end-labelled primers, the genomic DNA sample was amplified by PCR under standard conditions as follows: 200 ng genomic DNA from the affected patient as template, 0.2 mM dATP, dCTP, dGTP and dUTP, 6 pmol of the labelled diagnostic primer and non-labeled primer and 1 U of Taq polymerase bringing the total volume to 20 µl. Annealing temperatures and $MgCl_2$ concentrations varied with each different amplification (data not shown). PCR amplification using labelled deoxyribonucleotides was carried out as follows: 100 ng genomic DNA from the affected patient as template, 0.02 mM dATP, dCTP, dGTP and dUTP, 1 µCi

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[α-^{32}P]dCTP, 3 pmol of both primers and 1 U of Taq polymerase bringing the total volume to 10 µl.

Glycosylase mediated cleavage of DNA

The reaction mixture bearing the amplified target nucleic acid is then treated with Exonuclease I (ExoI) (Amersham Life Sciences) to digest the primers not extended in the amplification step. This is achieved by incubating 3 µl of the PCR reaction with 0.5 U of ExoI at 37°C for 30 min. The exonuclease was subsequently heat inactivated by incubating the reaction at 80°C for 15 min.

Escherichia coli UDG (0.5 U) (New England Biolabs) was then added and the incubation continued at 37°C for 30 min. Following treatment with UDG, the AP sites generated in the amplified product were cleaved to completion by adding NaOH to a final concentration of 0.05 M and heating the mixture for 15 min at 95°C. Under these conditions, cleavage occurs on the 5′ side of each AP site. The reaction was then neutralised by addition of Tris base to 30 mM final concentration. Both ExoI and UDG are diluted in buffer containing 0.07 M HEPES–KOH, pH 8.0, 1 mM EDTA, 1 mM DTT and 50% glycerol.

An equal volume of formamide loading dye (90% formamide, 0.025% Bromophenol blue, 0.025% Xylene cylanol) was added to the sample which was then heated at 85°C for 5 min. The sample was then loaded onto a 20% denaturing (7 M urea) polyacrylamide gel and electrophoresis was carried out for 3–4 h at 400 V for size analysis of the cleaved products in the sample. Following electrophoresis, autoradiography was carried out by exposing the gel directly to X-ray photographic film for 12 h at –70°C.

Solid phase GMPD

Solid phase detection of mutations was carried out as follows: PCR amplification using labelled deoxyribonucleotides and a biotinylated primer was carried out using 100 ng genomic DNA from each individual as template, 0.2 mM dATP, dGTP and dUTP, 0.02 mM dCTP and 1 µCi [α-^{32}P]dCTP, 3 pmol of both primers and 1 U of Taq polymerase bringing the total volume to 10 µl. The amplified DNA was treated with ExoI as described above. The sample was then divided into two aliquots (5.5 µl) and 1 µl UDG (0.5 U/µl) was added to one of the aliquots (A) and 1 µl H_2O added to the other (B) and both were incubated at 37°C for 30 min. An aliquot (1 µl) of NaOH was then added (0.15 M final) and the samples were incubated at 95°C for 15 min. The reaction was then neutralised by addition of 4.55 µl of 0.2 M HCl and the samples brought to 37 µl final volume and 1 M NaCl concentration upon addition of NaCl and TE buffer. The immobilisation was carried out using streptavidin coated beads (Dynabeads M-280 Streptavidin). Three µl of washed beads (10 mg beads/ml, washed in 1× B+W buffer, i.e. 10 mM Tris–HCl, pH 7.5, 1 mM EDTA and 1 M NaCl according to manufacturer's instructions) was added to each sample and incubated at room temperature for 5 min. The beads were then immobilised using a Dynal MPC-E (Magnetic Particle Concentrator for microfuge tubes) and the supernatant discarded. The beads were incubated with 40 µl 0.15 M NaOH and incubated at room temperature for 5 min. The supernatant was removed using MPC-E. The immobilised single stranded biotinylated DNA was then washed with 40 µl 1× B+W buffer and subsequently resuspended in 100 µl H_2O. The amount of ^{32}P remaining attached to the beads was quantified by Cerenkov scintillation counting. The results are expressed as a percentage and calculated as the amount of radioactive DNA remaining bound following GMPD processing (c.p.m. in A) divided by the total amount of radioactive DNA bound for each sample (c.p.m. in B).

RESULTS

Glycosylase mediated polymorphism detection (GMPD)

The GMPD process presented involves the use of UDG to detect the presence or absence of a uracil residue at a specific location in an amplified DNA molecule. A schematic diagram of the process is shown in Figure 1. The objective is to determine if a particular mutation (C to T) is present or absent at a specific location in a DNA test sample. PCR amplification of the sample is carried out using dGTP, dATP, dCTP and dUTP. The upper primer is labelled and serves as the diagnostic primer here. It is designed so that when it is extended during the amplification reaction, the first dUTP residue incorporated into the extended primer is at the mutation site if the mutation is present, or distal to the site if the mutation is absent. The oligonucleotide primers contain dG, dA, dT and dC and consequently are resistant to cleavage by UDG. Following PCR amplification of the test sample, the uracil bases are released by incubation of the amplified product with UDG generating AP sites. The phosphate linkages of the DNA at the resulting AP sites are then cleaved chemically or enzymatically. If a uracil base is present at the mutation site, the labelled upper strand will be cleaved to a specific length i.e., Yn, determined by the distance between the primer and the mutation site. If a uracil base is absent at the site, the labelled strand will be cleaved at the next position 3′ of the site where the first uracil is incorporated. Therefore, depending on whether a uracil base is present or absent at the mutation site, the extended labelled primer will be cleaved at, or 3′ of the site, and result in labelled fragments that can be distinguished by size (Xn or Yn). Such a size difference can readily be detected by existing DNA sizing technologies such as denaturing polyacrylamide gel electrophoresis and autoradiography or fluorescence imaging. In the schematic diagram shown, the presence of a band of Yn in length identifies the presence of the C to T mutation whereas the Xn band identifies the normal allele. A sample displaying both bands of Xn and Yn identifies the heterozygous state (Fig. 1).

Detection of the G1021A mutation in *RYR1* gene

We have used the GMPD process to detect a common point mutation (a G to A transition at position 1021) in the ryanodine receptor gene (RYR1) in the autosomal dominant disorder malignant hyperthermia (MH). MH predisposes individuals to an adverse reaction to the common inhalational anaesthetics agents and is potentially fatal (9). Patients are classified as MH susceptible (MHS) or MH normal (MHN) based on the response of skeletal muscle samples in an *in vitro* contracture test to caffeine and the anaesthetic halothane. The GMPD process was used here to detect the RYR1 G1021A in a well characterised MHS pedigree (10). As the G1021A mutation does not cause the gain or loss of a restriction site, it was previously shown to segregate with MH susceptibility in this pedigree by SSCP analysis (11). The sequence of the RYR1 gene from nt 1001 to 1044 is shown in Figure 2A. In this case the mutation results in a G to A base change in the upper strand at position 1021 and a corresponding C to T base change in the lower strand. Two

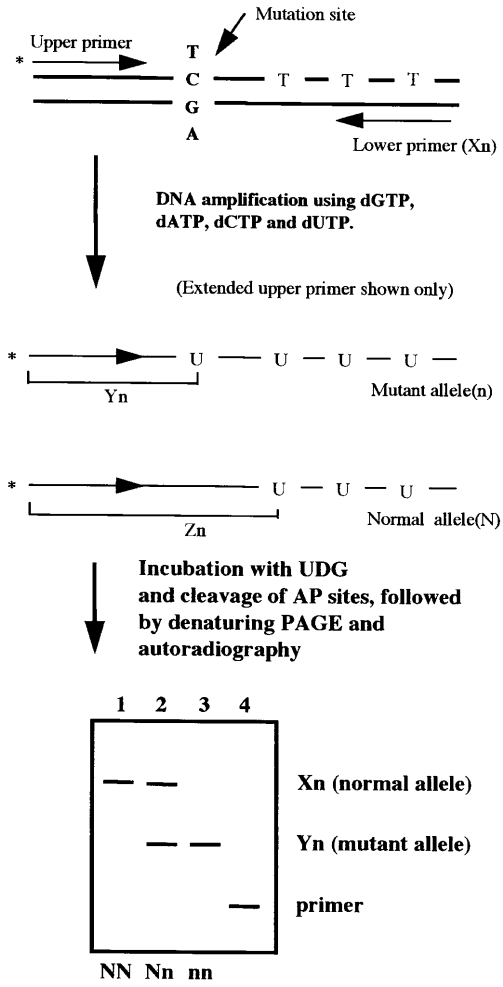

Figure 1. Schematic representation of the GMPD process using a labelled (*) upper (diagnostic) and unlabelled lower primer. Primers are denoted by arrows. The mutation site (C/G to T/A) is shown in bold type. Sites of dUMP incorporation during extension of the diagnostic primer are shown for the upper strand only and denoted by 'U'.

primers were designed to amplify the mutated region as a 44 bp DNA segment. The lower primer (21 nt) was designed so that in the mutant allele, there is an extension of 2 nt before the incorporation of the first dUMP at position 1021 while in the normal allele the first dUMP to be incorporated is 5 nt downstream of the 3′-end of this primer at position 1019. The lower primer was ^{32}P end-labelled and served as the diagnostic primer. Thus, amplification of the region followed by analysis of the fragments generated by UDG mediated cleavage should allow detection of the normal and mutant alleles as 25 and 23 nt fragments respectively. Figure 2B shows the result of the analysis of the members of the affected family. Before digestion with uracil DNA-glycosylase and AP site cleavage, the PCR products were incubated with ExoI. This is a single strand specific exonuclease and digests any unused primer including any non-specific single stranded DNA present. This can be seen by comparing lanes 1–8 with lane 10 that was not treated with *Exo*I. MHS individuals 1, 4, 7 and 8 display a 25 and a 23 nt fragment, showing that these individuals are heterozygous for the mutation. The 25 nt fragment only, is present in MHN individuals 2, 3, 5 and

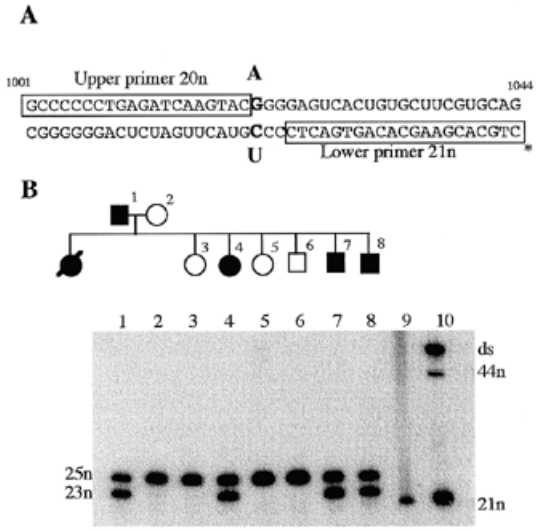

Figure 2. GMPD of the MHS *RYR1* G1021A mutation. (**A**) DNA sequence of the wild-type *RYR1* gene from nt 1001 to 1044. Primers used in amplification of this region are enclosed in a box. The asterisks denotes the end labelled diagnostic primer. The G1021A mutation site (G/C to A/T) is indicated by larger bold type. (**B**) Analysis of fragments generated from the RYR1 region 1001–1044 amplified from members of a MH pedigree following UDG mediated cleavage. In the pedigree, open circles and squares denote MHN individuals and closed circles and squares denote MHS individuals. Samples were subjected to denaturing polyacrylamide gel electrophoresis and visualised by autoradiography. Lane 9 shows the labelled diagnostic primer. Lane 10 shows the full length PCR product prior to cleavage. The band denoted 'ds' is due some of the undigested PCR remaining in the double stranded form

6 showing that they are homozygous for the normal allele (Fig. 2B). Results from the GMPD correspond with detection of the G1021A mutation in this family by direct DNA sequencing and SSCP analysis (10).

Detection of CFTR ΔF508 deletion mutation using the GMPD process

Insertion and deletion type mutations also occur commonly in genetic disease. The GMPD process can also be used for detection of these types of mutations and its application is shown here for analysis of individuals homozygous and heterozygous for the common CTT deletion mutation (ΔF508) in the CFTR gene in cystic fibrosis (12,13). The DNA sequence surrounding the site of this mutation is shown in Figure 3A. Two primers were chosen to amplify a 54 bp section of the CFTR gene encompassing the ΔF508 (deletion of CTT) mutation by PCR. In this case, we chose the lower primer as the diagnostic primer. Analysis of the amplified 54 bp product from a homozygous normal individual following glycosylase mediated cleavage generates a 34 nt fragment while a 31 nt fragment is present in the homozygous affected individual. Both the 34 and 31 nt fragments are present in the heterozygous carrier (Fig. 3B, lanes 1, 2 and 3). The ΔF508 deletion can also be detected using end-labelled upper primer and will produce a 22 and a 21 nt fragment for the normal and mutant alleles respectively (data not shown). An alternative labelling approach for detecting the normal and mutant alleles was also used i.e., internal labelling by incorporation of an [α-^{32}P]dNTP during DNA amplification. In this case, [α-^{32}P]dCTP was utilised, therefore, during extension of the primers, the 54 bp PCR

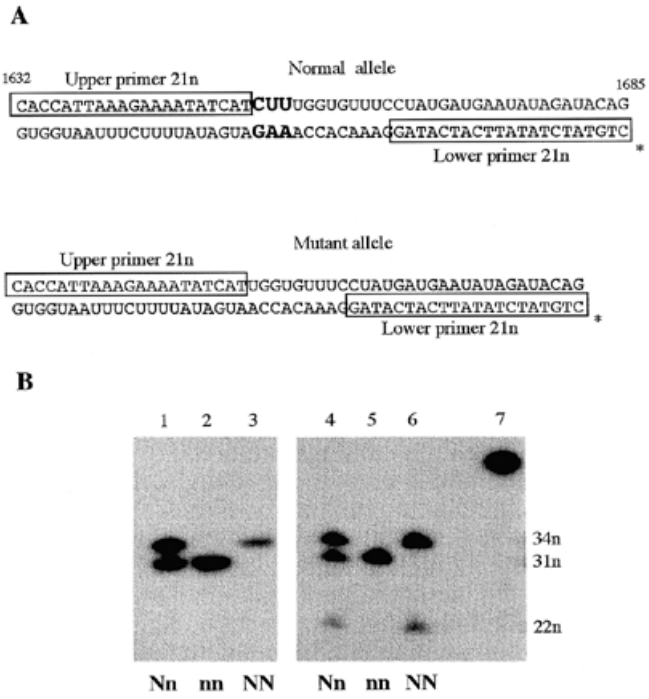

Figure 3. GMPD of the CFTR ΔF508 mutation. (**A**) Sequence of PCR amplified region of wild-type *CFTR* gene from nt 1632 to 1685. Primers used for amplification of this region are enclosed in a box. The asterisks denote the ^{32}P end-labelled diagnostic primer. The ΔF508 mutation site (deletion of CTT) is indicated by larger bold type. (**B**) Analysis of end labelled fragments generated from the amplified CFTR region 1632–1685 from a CF heterozygous carrier (Nn, lane 1), a CF homozygous affected (nn, lane 2) and homozygous normal (NN, lane 3) individual following UDG mediated cleavage. Analysis of internally labelled (using [α-^{32}P]dCTP) fragments for the heterozygous (lane 4), homozygous affected (lane 5) and homozygous normal (lane 6) individuals. The full length PCR product (54 nt) is shown in lane 7.

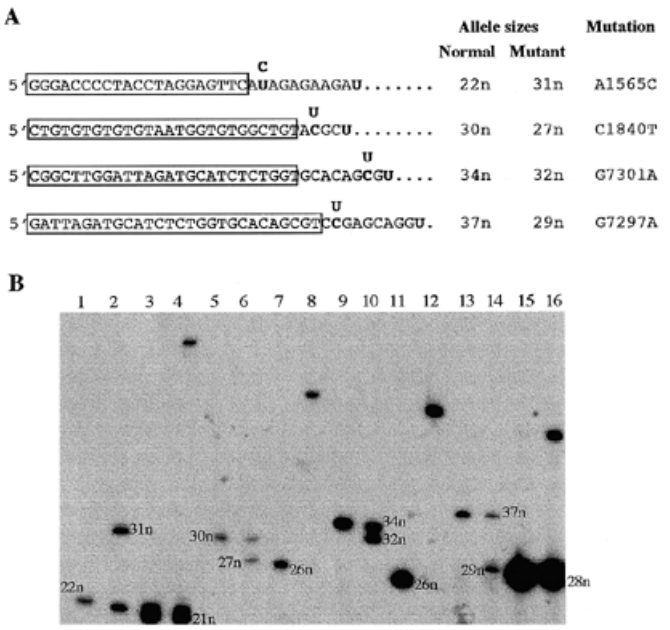

Figure 4. GMPD of MHS *RYR1* A1565C, C1840T, G7301A and G7297A mutations. (**A**) The labelled diagnostic primers for each mutation are enclosed in a box. The sites of dUMP incorporation during PCR amplification are shown in bold type along with the corresponding sizes of the expected diagnostic fragments. (**B**) Untreated PCR amplification products of regions surrounding each mutation are shown in lanes 4, 8, 12 and 16 respectively. The labelled diagnostic primers for each mutation are shown in lanes 3, 7, 11 and 15 (21, 26, 26 and 28 nt, respectively). Lanes 1, 5, 9 and 13 shows the UDG mediated cleavage products generated from the amplified DNA from MHN individuals (22, 30, 34 and 37 nt, respectively). Lanes 2, 6, 10 and 14 shows the cleavage products from MHS individuals (31, 27, 32 and 29 nt, respectively). The upper and lower primer pairs for each mutation respectively are as follows (numbers refer to nucleotide position within *RYR1* gene): 1486–1506 and 1567–1576 (in addition to the following intronic sequence at the 5′-end of the primer, 5′-GGGACCCCTAC-3′); 1813–1838 and 1858–1881; 7270–7293 and 7308–7333; 7270–7293 and 7299–7326.

product was labelled at cytosine residues. Following analysis of the glycosylase mediated cleavage products, all fragments containing C residues will be detected. Individuals carrying two normal alleles display bands of 34 and 22 nt (Fig. 3B, lane 6). An individual homozygous for the mutation has a band of 31 nt (Fig. 3B, lane 5) while the heterozygous carrier displays a 34, 31 and 22 nt band (Fig. 3B, lane 4). All samples contained smaller labelled DNA fragments (<5 nt) which ran off the gel. The 22 nt band appears less intense than the 31 or 34 nt fragments. This is due to the different amounts of [α-^{32}P]dCMP residues in each fragment. The 22 nt fragment has one C residue whereas the 31 and 34 nt fragments have three C residues.

GMPD of various MHS mutations within different sequence contexts

In order to further validate the use of the GMPD for generalised mutation detection we demonstrate its use for the detection of four additional RYR1 point mutations (A1565C, C1840T, G7301A and G7297A) (reviewed in 9) in MHS. The sequence of the extended diagnostic primers and the expected sizes of the normal and mutant alleles for each mutation are shown (Fig. 4A). A MHN and MHS sample for each of the above mutations was analysed by GMPD. The normal alleles [22, 30, 34 and 37 nt (Fig. 4B, lanes 1, 5, 9 and 13) for the A1565C, C1840T, G7301A and G7297A, respectively] were detected in all individuals whereas the expected mutant alleles [31, 27, 32 and 29 nt (Fig. 4B, lanes 2, 6, 10 and 14) for the A1565C, C1840T, G7301A and G7297A, respectively] were observed in the different heterozygous MHS individuals.

Allele size design

Tailoring of the size of the mutant and normal alleles generated in the GMPD process would facilitate multiplex detection of mutations. The use of mismatches and inosine residues in the non-diagnostic primer in order to change the size of the normal allele was investigated. Inosines in the template strand usually direct the incorporation of cytosine residues into the newly synthesised strand (14,15). The example shown is for the G1021A mutation in MHS. Specifically, we positioned the non-diagnostic primer so that there were no adenine residues between the 3′-end of the primer and mutation site. Some or all of the adenine residues within the primer were also replaced with inosine residues or with a mismatched base. Replacement of the penultimate 3′ adenine base in the non-diagnostic primer (upper

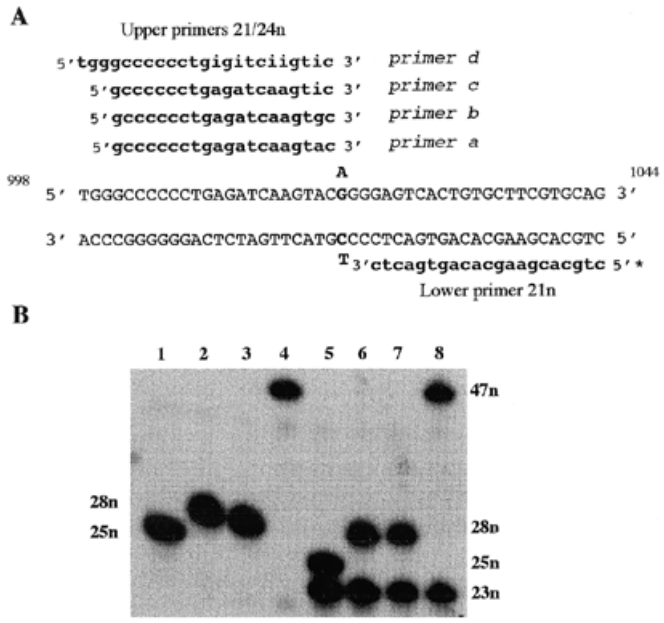

Figure 5. Tailoring of the non-diagnostic primer. (**A**) DNA sequence of the wild-type RYR1 gene from nucleotide 998 to 1044. Primers used in amplification of this region are shown in lower case letters. The asterisk denotes the end labelled diagnostic primer (lower primer). The G1021A mutation site (G/C to A/T) is indicated by larger bold type. Primers a, b, c and d are the various uppers primers used in the amplification reaction and contain: no inosines, a 'g' mismatched nucleotide, one inosine or five inosine residues respectively. All upper primers are 20 nt in length except for primer d which is 23 nt. (**B**) Analysis of fragments generated from the amplified RYR1 region 998/1001–1044 from an MHN (lanes 1–4) and MHS (lanes 5–8) individual following UDG mediated cleavage. The upper primer used in the amplification reactions was primer a (lanes 1 and 5), primer b (lanes 2 and 6), primer c (lanes 3 and 7) and primer d (lanes 4 and 8).

primer) with guanine or inosine increased the length of the normal allele from 25 to 28 nt following glycosylase mediated cleavage of the 47 bp amplified product (Fig. 5). Replacement of all the adenines in the non-diagnostic primer with inosines yielded a labelled normal allele of 47 nt that is devoid of uracil bases and resistant to cleavage mediated by UDG (Fig. 5B, lane 4). By contrast, the corresponding untreated mutant allele has a single uracil in the entire 47 nt labelled strand and yields a 23 nt fragment after UDG mediated cleavage (Fig. 5B, lane 8). This example serves to illustrate the flexibility of primer design that is permitted with GMPD. Importantly, the example also shows that uracil is not incorporated opposite inosine residues in the template stand during the amplification reaction, since following UDG mediated cleavage, only the full size normal allele is detected if the five adenines in the non-diagnostic primer are replaced with inosines (Fig. 5B, lanes 4 and 8).

Solid phase GMPD

The flexibility of primer design when using GMPD and the fact that a glycosylase resistant product from the normal allele and a glycosylase sensitive product from the mutant allele could be amplified readily prompted us to investigate the possibility of adapting GMPD of the G1021A mutation to solid phase.

In this case, the lower primer was as shown in Figure 5 except that it was biotinylated and moved 2 nt closer to the 1021 position so that the 3′-end of the primer was next to the mutation site. This primer was used with primer d (Fig. 5; all adenine replaced by inosines) to amplify a 47 bp product encompassing the RYR1 G1021A mutation site from the MHN and MHS (shown to carry the G1021A mutation) members in the pedigree shown in Figure 2. [α-^{32}P]dCTP was included in the amplification reaction. The [α-^{32}P]dCTP is incorporated into both amplified strands but importantly can only be incorporated at, or 3′ of the mutation site in the biotinylated strand. Following amplification, half of the amplified 47 bp product was treated with UDG while the remaining half of each sample was treated in an identical manner except that UDG was omitted. Following this, the biotinylated strand was isolated and disassociated from its complementary strand using streptavidin coated magnetic beads and alkali treatment. The radioactivity remaining attached to the beads was quantified by Cerenkov counting and the results are expressed as the percentage of total radioactive DNA remaining bound (Table 1). In all cases, there was a clear difference between the MHN and MHS samples. The percentage DNA remaining bound for MHN samples varied from 90 to 99%, while MHS samples varied from 36 to 52%. It is not clear why there is a variation of up to 16% in the assay. This may reflect variation in the amplification of the normal and mutant alleles or sampling error. Nonetheless, even with a 16% variation, there is a clear difference between the normal and mutant alleles.

Table 1.

Family member identification no.[a]	Disease status	Genotype[b]	% Radioactivity bound[c]
1	MHS	– / +	40
2	MHN	– / –	97
3	MHN	– / –	99
4	MHS	– / +	36
5	MHN	– / –	90
6	MHN	– / –	91
7	MHS	– / +	40
8	MHS	– / +	52

[a]no. refers to the family member shown in the pedigree in Figure 2B.
[b]Genotype with respect to absence or presence of the RYR1 G1021A mutation as determined by SSCP analysis.
[c]Percentage radioactivity bound is calculated as the amount of radioactivity remaining bound to the beads following UDG mediated cleavage of the amplified DNA divided by the amount of radioactivity remaining bound to the beads when the amplified DNA is not treated with UDG.

DISCUSSION

In this work we describe a novel process for identification of mutations in DNA which exploits the DNA repair enzyme UDG. This process relies on the ability of UDG to specifically excise U residues incorporated into a mutation site in amplified DNA. Twelve possible point mutations can occur in DNA. Ten of these result in the loss or gain of a T (U) residue. The remaining two point mutations G to C and C to G do not involve a T residue. The most common type of point mutations (and polymorphisms) in human genetic disease result from deamination of C or 5 methyl

C residues to U or T respectively, thus, C to T transitions account for a high proportion of single point mutations (16,17). Deletion and insertion mutations also occur frequently in DNA and all can be detected by using GMPD. Thus, the GMPD process described offers a means to detect most mutations in a streamlined process with single enzyme. To detect all known mutations, it will be necessary to detect G to C and C to G mutations. Utilisation of a DNA-glycosylase that recognises a substrate base incorporated in place of cytosine or guanine residues should readily allow detection of G to C and C to G substitutions and a second DNA-glycosylase is currently being evaluated for this purpose. However, UDG alone is sufficient for detection of the majority of mutations.

In this work, we have described the use of GMPD for detection of several different point mutations and a deletion mutation demonstrating that the method works well with different mutations in different sequence contexts. The GMPD process is also very flexible and versatile and facilitates multiplex mutation analysis since large or small amplification products can be utilised and the sizes of cleavage products can be designed to suit ones requirements by appropriate tailoring of the diagnostic and non-diagnostic primers. Furthermore, differential fluorescent dyes in conjunction with differential diagnostic primer sizes should allow the analysis of multiple mutations per electrophoresis lane. Replacement of adenines with inosines in a primer exclude incorporation of uracil residues in the newly synthesised DNA complementary to the primer strand. This facilitates solid phase GMPD since UDG resistant or sensitive strands of DNA can be amplified dependent on presence or absence of the incorporation of a uracil residue at the mutation site. We have successfully demonstrated such a solid phase approach for detection of the G1021A mutation in RYR1 gene of an MH pedigree. Although the number of samples tested here was small, clear differences were observed between normal and mutant alleles illustrating the potential of this approach for large scale screening of known mutations.

The GMPD method has many attractive features which offers significant advantages over several current technologies utilised for detection of known mutations such as direct DNA sequencing, restriction enzyme digestion (PCR-RFLPs), single strand conformation analysis (SSCP), ligase chain reaction, allele specific oligonucleotide probes, ARMS test and competitive oligonucleotide priming (18–22). In particular, GMPD offers the specificity of direct DNA sequencing but is a much simpler process with almost all mutations detected using a single enzyme. The method does not rely on heteroduplex formation but works equally well on homozygous and heterozygous samples. The annealing of primers is not dependent on stringent or differential hybridisation conditions. The amplification of samples using dUTP works well with several thermostable polymerases and overall only sequence information of 20–25 nt on either side of the mutation site is required in order to design appropriate primers for the amplification process. The process can be performed directly on any DNA sample and the process is robust and requires relatively few manipulations. Solid phase GMPD using one of the many applicable detection systems avoids the use of time consuming gel electrophoresis and will allow rapid and high throughput sample analysis.

The great increase in our knowledge of the human genome is a relatively recent event, with the generation of successful genome wide genetic maps (23,24). The availability of such dense maps have encouraged many studies including genetic linkage of mono- and polygenic traits. For future investigations of polygenic traits and for generation of a human genetic map based on biallelic polymorphisms, it will be necessary to genotype large numbers of simple single nucleotide polymorphisms (SNP) spread throughout the entire genome. The specificity, versatility and simplicity of the GMPD technique should make it an attractive candidate for both small and large scale application in the mutation detection and polymorphism genotyping fields.

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