Mismatch Cleavage Detects Base Deletion in Cystic Fibrosis Gene

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ABSTRACT

The ΔF508 is the most common defect in the cystic fibrosis (CF) gene; it involves in a 3-base deletion in codon 508 and results in the loss of a phenylalanine residue at amino acid position 508. Our previous results have shown the mismatch enzyme cleavage at the mismatch of a DNA duplex in identifying a specific DNA sequence or a point mutation. The assay is simple and reliable. By manipulating the melting temperature (Tm) for the hybrids of the DNA targets and the deoxynucleotide probes, the mismatch cleavage assays are able to detect the most common defective CF gene, ΔF508. The assays with a ΔF508 and a normal wild-type probe can differentiate the three genotypes, i.e., ΔF508/ΔF508, ΔF508/normal and normal/normal. Furthermore, the addition of ammonium acetate amplifier to the assay for recycling the target DNA can increase the sensitivity to a level that is sufficient to detect the mutated target in a few micrograms of genomic DNA without the aid of PCR amplification. The detection of the base deletion, the amplification of sensitivity and the differentiation among the genotypes of normal, carrier ΔF508 and mutant ΔF508 suggest the useful application of mismatch cleavage in genetic diagnosis at the DNA level.

INTRODUCTION

Cystic fibrosis (CF) is an inherited disorder characterized by progressive lung disease, pancreatic insufficiency, impaired growth, elevated sweat electrolyte values and other less common clinical findings (4,9). The gene and its DNA sequence have been characterized (9,16). A mutant allele frequency of 0.022 in the United States was derived from the autosomal recessive mode of inheritance (1). Although the frequency of cystic fibrosis is not uniformly high among all Caucasian populations, a consensus estimate is that it occurs once in 2000 live births. Approximately 70% of the CF gene mutations in cystic fibrosis patients correspond to a specific deletion of three base pairs (ΔF508), which results in the loss of a phenylalanine residue at amino acid position 508 (8). A simple and reliable test to identify mutations in the CF gene has important implications for genetic diagnosis at the DNA level. Although the amplification of polymerase chain reaction (PCR) coupled with DNA sequencing has been the most definitive procedure for identifying abnormal DNA sequences, it remains labor-intensive and expensive for clinical uses (17,18). Several alternative approaches have been developed for screening or detecting DNA mutations (2,3,8,10,11,13–15). Kerem et al. have demonstrated the detection of ΔF508 by oligonucleotide dot blot hybridization (8). Since our first demonstration of the specific cleavage of mismatch repair (MR) enzymes in determining a DNA point mutation (7,12), Lishanski et al. used the specific mutS binding to mismatched DNA heteroduplex and showed the resulting mobility shift for screening of the CF gene mutation (11). Recently, we cloned the mutY gene and overexpressed and purified the protein to near homogeneity for the assay (5,19). This purified mutY enzyme is free of exonuclease activity and does not cut single-stranded (ss) DNA. In addition to determining a point mutation, the specific cleavages at the mismatch of a known probe will also reveal a specific complementary nucleic acid sequence in the target DNA samples. The addition of ammonium acetate (AA) amplifiers in the assay for recycling the target DNA further increases the assay sensitivity (6). This study demonstrates a modified procedure that detects the base deletion for ΔF508. The amplification of sensitivity and the differentiation for the three genotype groups are discussed.

MATERIALS AND METHODS

Enzymes, Chemicals and Reagent

MutY MR enzyme was overproduced in an E. coli HMS174 host that harbored a mutY overexpression vector. The procedures of preparation, isolation and purification of MutY protein were described previously (19).

The Sephadex® G-25 Quick Spin™ Column, Tris, EDTA, sodium dodecyl sulfate (SDS) and T4 Polynucleotide Kinase (PNK) were purchased from Boehringer Mannheim (Indianapolis, IN, USA), [α-32P]ATP (3000 Ci/mmol) and acrylamide were obtained from Amersham Pharmacia Biotech (Piscat-
hybridize the probe (lanes 4–6) is a 21-base oligomer that will target DNA sequence, except the oxynucleotide probe is complementary to target DNA sequence, except the base to be removed.

Table 1. Sizes, Sequences and Tm of the Probes and the Targets

<table>
<thead>
<tr>
<th>Property of Target and Probe</th>
<th>Normal Target</th>
<th>ΔF508 Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>222 base</td>
<td>219 base</td>
</tr>
<tr>
<td>PCR primers for target preparation</td>
<td>left 5'-GACCATTAAGAATATGAT-3'</td>
<td>ΔF508 probe (21 bp)</td>
</tr>
<tr>
<td></td>
<td>right 5'-CATTCACAGTAGCTTACCCA-3'</td>
<td></td>
</tr>
<tr>
<td>Probe (size) for detection</td>
<td>WT probe (19 bp)</td>
<td></td>
</tr>
<tr>
<td>Sequence of probe</td>
<td>5'-TAGGA,AAACA,CAAAATG-3'</td>
<td>5'-ATAGG,AAACA,AGAAATGAT,TT-3'</td>
</tr>
<tr>
<td>Tm of ΔF508 probe/target</td>
<td>30°C</td>
<td>47°C</td>
</tr>
<tr>
<td>Tm of WT probe/target</td>
<td>44°C</td>
<td>27°C</td>
</tr>
<tr>
<td>Tm for cleaved probe fragments</td>
<td>28°C and 26°C</td>
<td>24°C and 26°C</td>
</tr>
</tbody>
</table>

`a`These 2 primers are used in amplification for both normal and ΔF508 DNA targets. A is the site of the mismatch and the base to be removed.

`b`Tm (°C) for the probe/target DNA duplex with one mismatch was obtained from OLIGO data base (NBI).

`c`Tm (°C) was calculated by 2(A + T) + 4(G + C) method for DNA duplex.

Figure 1. Detection and differentiation of the normal CF DNA and the mutated ΔF508 DNA by MutY enzyme cleavage. The target DNA fragments (ca. 220 bp) were prepared by PCR amplification of genomic DNAs from patients with (i) mutated ΔF508ΔF508, lanes 1 and 4) or (ii) carrier (ΔF508/normal, lanes 2 and 5) and normal (lanes 3 and 6) CF genes. The ΔF508 gene has a 3-base deletion at codon 508 (ΔF508). The carrier contains a mutated ΔF508 allele and a normal allele and is a heterozygote. The ΔF508 probe (lanes 4–6) is a 21-base oligomer that will hybridize the ΔF508 DNA target to generate a G/A mispair with the A at the probe. The WT probe (lanes 1–3) is a 19-base oligomer that will generate a G/A mismatch with the normal DNA target. In the assay, approximately 100 fmol of 32P-labeled probe and 5 fmol of the target DNA were incubated at 37°C for 5 h with 20 ng cloned MutY enzyme. After the assay, the probes and the cleaved fragments were separated by gel electrophoresis and visualized in the autoradiograph for determining the site of cleavage. M11 is an 11-bp marker.

The specific cleavage of G/A mismatch at A by MR enzymes and the expected size of the cleaved DNA fragment identify the target DNA sequence. The mismatches were generated by annealing the 32P-labeled probe and the target DNA in MutY assay buffer (5, 19). The mixture was heated at 90°C for 3 min and then cooled slowly to room temperature to generate DNA heteroduplexes with a G/A mismatch.

In the assay, approximately 10000–20000 disintegrations per minute (dpm) of the 32P-labeled heteroduplex substrate were incubated overnight (ca. 15 h unless specified otherwise in the text) at 37°C with 50 ng of the MutY enzyme preparation in 20 µL of assay away, NJ, USA) and Bio-Rad (Hercules, CA, USA), respectively.

Probes and Targets

The target DNA fragments were prepared by PCR amplification of genomic DNAs previously characterized as homozygous wild-type (WT), homozygous or heterozygous for the ΔF508 mutation (4). Table 1 lists the primers for PCR preparations of the 219- or 222-bp CF target. The two oligomer probes were purchased from Cruachem (Sterling, VA, USA), and Table 1 lists their sequences. The deoxynucleotide probe is complementary to target DNA sequence, except the “A” in bold type with underline. The probe will hybridize the target DNA and generate a G/A mismatch. The A for cleavage is located usually near the center of the probe. Table 1 also lists the melting temperatures (Tm) for the hybrids of the targets and the products. The DNA probes that contained the A for cleavage were 32P-labeled at the 5' end by T4 PNK reaction. For the reaction, approximately 2–3 pmol of [r-32P]ATP and 5–10 pmol of synthesized deoxynucleotide were incubated at 37°C for 1 h with 20 U of PNK in 20 µL of Tris buffer (pH 7.8) provided by the manufacturer (Boehringer Mannheim). The labeled DNA was purified with a Sephadex G-25 Quick Spin Column, according to the procedure of the manufacturer.

Enzyme Assay for Detection of Target DNA

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buffer (20 mM Tris, pH 7.6, 1 mM EDTA and 50 µg of bovine serum albumin [BSA] per mL). An equal volume of the dye solution was added to a 3-µL aliquot of the assay mixture to stop the reaction before electrophoresis on a 20% acrylamide gel.

RESULTS AND DISCUSSION

The ΔF508 is the most common mutant of the CF gene that involves a specific 3-base deletion. Approximately 70% of the CF mutations have the 3-base deletion. During the last few years, we have used the specific cleavage at G/A or G/T mismatch by MR enzymes in the detection of DNA point mutations (5,19). The enzymes are very specific and only cut the mispaired DNA duplex at A or T (5,7,19). For practical purposes, the ΔF508 is a good model for developing mismatch cleavage assay in determining deletion mutation.

In experiments with this model, three CF DNA targets of approximate 220 bp were prepared by PCR amplification of genomic DNA from normal and CF patients with ΔF508 in one or both alleles. We also manipulated the Tm of the hybrids and designed the procedure such that at the 37°C assay temperature, the ΔF508 probe will anneal the ΔF508 DNA target but not the normal target. Conversely, the WT probe hybridizes the normal target but not the ΔF508 target. Table 1 summarizes the sizes, sequences and Tm's of the probes and the CF targets. Based on the estimation of the OLIGO™ database (National Biosciences Inc. [NBI], Plymouth, MA, USA), one mismatch will reduce the Tm of DNA duplex by 5°–6°C.

During the assay at 37°C, the 19-bp WT probe annealed the normal target and generated a G/A mismatch for cleavage at A. However, this probe and the ΔF508 target would have 4 mismatches that reduce the Tm to about 27°C. Therefore, this WT probe does not hybridize the ΔF508 target in 37°C solution and remains as ssDNA. Because MutY does not cut ssDNA, Figure 1, lane 1 shows no cleavage for this probe. This WT probe did hybridize the normal CF targets that had 2 normal CF alleles (lane 3) or one normal CF allele (lane 2). The expected 10-bp DNA fragment of the cleaved product was observed in lanes 2 and 3. Likewise, the experiment with the 21-bp ΔF508 probe that has a mismatch with the ΔF508 target but has 4 mismatches with the normal target, demonstrated a predicted similar result in Figure 1, lanes 4 and 5. Only the sample in lane 4 with ΔF508 target in both alleles and the sample in lane 5 with one ΔF508 allele anneal the ΔF508 probe, resulting in the cleavage of G/A mismatch and the appearance of the expected 11-bp product. The cleaved products with expected sizes in Figure 1 indicate that the cleavage of the WT probe (lanes 2 and 3) at G/A mismatch occurs only in the assay containing the normal target; whereas, the cleavage of the ΔF508 probe (lanes 4 and 5) occurs only in the presence of mutant ΔF508 target.

To evaluate the sensitivity, the assays were carried out with approximately 100 fmol of WT or ΔF508 probe and decreasing quantities of the normal and the ΔF508 targets. The results in Figure 2 show that the MutY enzyme does not cut single-stranded probe (lane C). Figure 2, lanes 1–3 and 4–6, further shows that the cleaved products decrease with reducing amounts of DNA target in the assay mixtures. The lowest detection limit is about 1 fmol (Figure 2, lanes 3 and 5). If one genomic DNA molecule has 3 × 10⁹ bases in 10⁵ genes, 1 fmol will be approximately 10¹² fg or 1 mg genomic DNA. The assay that requires 1 mg of genomic DNA in 20 µL incubation mixture is obviously impractical. In addition, the isolation of 1 mg DNA needs 0.3 g or more of human cells. To make simple diagnostic kits for genetic diagnosis, the assay would require a sensitivity able to detect 1 amol or less of the target (i.e., an increase of sensitivity 10³-fold from the present assay).

During the last 2–3 years, we have developed a procedure of recycling the DNA target for improving the sensitivity by including the amplifier in the assay. Figure 3 demonstrates the recy-

![Figure 2. Sensitivity in the assay of CF gene. Approximately 100 fmol of probe were incubated at 37°C with 5 (lanes 1 and 4), 1 (lanes 2 and 5), 0.2 (lanes 3 and 6) and 0 (lane C) fmol of target DNA in 20 µL of reaction mixture for 24 h. The cleaved fragments were determined from the autoradiogram following gel electrophoresis. M11 and M14 are 11- and 14-bp markers, respectively.](image1.png)

![Figure 3. Signal amplification of mismatch cleavage in detection of ΔF508 gene. Approximately 3 fmol of ΔF508 target was incubated with 100 fmol of 3²P-labeled probe and 50 ng MutY at 37°C for 40 h (lane 1) as described in Materials and Methods. For amplifying the sensitivity, 25 fmol (lanes 2 and 2P) and 75 fmol (lanes 3 and 3P) of AA amplifier were included in the incubation mixtures. Lanes 2P and 3P showed the autoradiogram for 3-µL samples of assay solutions from lanes 2 and 3 pretreated in 1M piperidine at 90°C for 30 min before gel electrophoresis.](image2.png)
clinging of the target and the resulting amplification of mismatch cleavage in the presence of 25 mM (lanes 2 and 2P) or 75 mM (lanes 3 and 3P) of the AA amplifier. In the assay, the DNA hybrid of the probe and the target has a $T_m$ of 47°C and a G/A mismatch for cleavage by MutY. During the incubation at 37°C, the MutY enzyme will only cut the A of the G/A mismatch in the probe that anneals the target. In the assay with 3 fmol of target and 100 fmol ΔF508 probe, the excess, unhybridized probe remains as single-stranded oligomer and is not the substrate for cleavage. They will not be cut by MR enzymes. The A for the mismatch cleavage usually locates near the center of the probe. The cleavage at the mismatched A of the probe will split the probe in half and reduces the $T_m$ to approximately 24°–28°C. Because the $T_m$ is below the 37°C incubation temperature, the product will dissociate from the target and

Figure 4. Sensitivity of mutY detection with AA amplification for ΔF508 target. The assay was carried out with 0 fmol (lane 1, control), 7 fmol (lanes 2 and 3), 0.7 fmol (lanes 4 and 5), 0.07 fmol (lanes 6 and 7) and 0.007 fmol (lane 8) of ΔF508 target at 37°C for 20 h. Additional 75 mM of AA was included in lanes 1, 2, 4, 6 and 8. The rest of the assay mixture in lane 2 was incubated at 37°C for another 20 h and divided into 2 parts to repeat the experiment in Figure 3, lanes 2P and 3P, for quantifying mutY cleavage. An equal volume of stopping dye solution was added to the first half for gel electrophoresis (lane 9). The other half was pretreated with 1 M piperidine at 90°C for 30 min before adding to the dye solution for gel electrophoresis (lane 10).
free the target for another round of annealing and cleavage. However, this does not seem to occur, or if it does, occurs too slowly to be detected without the addition of amplifier (Figure 3, lane 1). Figure 3, lanes 2 and 2P, shows the increases of sensitivity by 10-fold to 20-fold in the presence of 25 mM amplifier. Figure 3, lanes 3 and 3P, further shows more amplification of sensitivity with 75 mM of amplifier. Approximately 100 fmol of probe were included in the assay. After a 40-h incubation, more than half (50 fmol) of the input probe were observed as cleaved products in the autoradiogram (lanes 3 and 3P). Since only 3 fmol of target were originally included in the assay, the target must have been used again and again many times (i.e., the amplifier frees the target for recycling). The increase of amplification with increasing concentrations of AA (lanes 2 and 3) is consistent with our previous results (6), supporting that AA competes with mutY for substrate binding sites and causes the release of target DNA for recycling. Once the substrate DNA binds to AA, the phosphodiester linkage can be broken at the 3′ or 5′ end or both ends of the deoxy(A). The possible mechanisms of mismatch cleavage and the amplification by amines were previously discussed (6).

Figure 4 further shows that AA at 75 mM recycles the target and increases the sensitivity. As low as a few attomoles of target were identified (lane 8). Because the lowest detection limit in lane 8 with amplifier is 7 amol, there is a $10^2$–$10^3$ improvement of sensitivity. Again, in the repeated experiment, the assay yields more product than the target and the uncut probe left in the reaction mixture (lanes 9 and 10). By further modifying the procedure with nonradioactive labels, it is possible to make simple diagnostic kits for testing defective genes at the DNA level without prior target amplification.

ACKNOWLEDGMENTS

This work was supported in part by EPA Grant No. R818104-01. This is contribution No. 3942 from the Pathobiology Department of the University of Maryland.

REFERENCES


Received 1 December 1997; accepted 16 March 1998.

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