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Mutation detection by mismatch binding protein, MutS, in amplified DNA: Application to the cystic fibrosis gene

(PCR amplification/DNA heteroduplexes/DNA-binding protein/mobility-shift assay)

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ABSTRACT **An experimental strategy for detecting heterozygosity in genomic DNA has been developed based on preferential binding of *Escherichia coli* MutS protein to DNA molecules containing mismatched bases. The binding was detected by a gel mobility-shift assay. This approach was tested by using as a model the most commonly occurring mutations within the cystic fibrosis (*CFTR*) gene. Genomic DNA samples were amplified with 5′-end-labeled primers that bracket the site of the ΔF508 3-bp deletion in exon 10 of the *CFTR* gene. The renatured PCR products from homozygotes produced homoduplexes; the PCR products from heterozygotes produced heteroduplexes and homoduplexes (1:1). MutS protein bound more strongly to heteroduplexes that correspond to heterozygous carriers of ΔF508 and contain a CTT or a GAA loop in one of the strands than to homoduplexes corresponding to homozygotes. The ability of MutS protein to detect heteroduplexes in PCR-amplified DNA extended to fragments ≈500 bp long. The method was also able to detect carriers of the point mutations in exon 11 of the *CFTR* gene by a preferential binding of MutS to single-base mismatches in PCR-amplified DNA.**

The rapid rate of discovery of disease genes increases the need for development of high through-put mutation detection methods allowing screening of many individuals at multiple loci. It has now become possible to detect point mutations by a variety of techniques, the most popular of which exploit conformational differences of mismatched heteroduplexes using denaturing gradient gel electrophoresis, or single-stranded conformational polymorphisms revealed by gel electrophoresis. These and other methods of mutation detection have been recently reviewed (1, 2).

An application of a mismatch-cleaving enzyme, MutY, for detection of single-base substitutions in human oncogenes has been described (3). This paper reflects a growing interest in DNA repair enzymes as potential tools for screening genomes for polymorphisms and disease-causing mutations. An excellent example of the power of such tools is the development of genomic mismatch screening (4). This procedure uses the *Escherichia coli* dam methylase-directed mismatch repair enzymes to clone regions of genomes that are identical by descent.

We used the ability of one member of this protein family, MutS protein, to recognize and bind mismatches in order to develop a technique that would test for heterozygosity of a locus in PCR-amplified DNA from an individual. *In vivo*, MutS protein initiates a series of events leading to a correction of replication errors (5, 6). This protein is able to bind various single-base mismatches in short synthetic oligonucleotides *in vitro* (7). We extended this finding to PCR products from two regions of the human cystic fibrosis (*CFTR*) gene that harbor the most frequently occurring mutations within this gene. The choice of the *CFTR* gene was motivated by the availability of a collection of well-characterized mutations and the importance of rapid screening methods for carrier status. We have established that MutS preferentially bound mismatched heteroduplexes formed upon reannealing of PCR product from genomic DNA heterozygous for certain frameshift and point mutations within the *CFTR* gene and that this ability is retained on PCR fragments as large as ≈500 bp.

MATERIALS AND METHODS

MutS protein was a gift from P. Modrich (Duke University Medical School). Genomic DNA samples extracted from the peripheral blood of patients at Oakland Children's Hospital were kindly provided by J. Johnson.

PCR Primer Design and End Labeling. PCR primers used in this study are listed in Fig. 1*a*. They were designed by using the PRIMER software except for the 5/6 and 10/11 pairs, which were chosen according to ref. 8. Oligonucleotides were purchased from Genosys (The Woodlands, TX) and 5′-end-labeled by using T4 polynucleotide kinase (Boehringer Mannheim) and [γ-^{32}P]ATP (10 mCi/ml; >5000 Ci/mmol; 1 Ci = 37 GBq; Amersham).

PCR Amplification. Amplifications were carried out in the MiniCycler (MJ Research, Cambridge, MA) or in the Perkin–Elmer 9600 thermocycler using 0.2-ml thin-walled tubes. The hot start technique with AmpliWax PCR gems was performed according to the procedure recommended by the manufacturer (Perkin–Elmer). Genomic DNA (250 ng) was used as a target. A total of 27 PCR cycles were performed consisting of a 1-min denaturation step at 94°C, a 1-min reannealing step at 58°C (55°C for the 5/6 and 52°C for the 10/11 primer sets), and a 1-min extension step at 74°C. The last cycle was followed by a 7-min final extension. Amplification was followed by denaturation (95°C; 3 min) and reannealing (67°C; 1 hr) of the PCR product. The last two steps were incorporated into the same program and were followed by storage at 4°C. Thermostable DNA polymerases used for DNA amplification were *Taq* (Perkin–Elmer) and *Pfu* (Stratagene).

MutS Protein Binding and Mobility-Shift Assays. The reannealed PCR product (2–6 μl) was mixed with 1–4 μl (≈4 pmol) of MutS and supplemented with the assay buffer (7) so that the total volume never exceeded 10 μl. In some experiments, 1 μg of bovine serum albumin was added to each reaction mixture. The mixtures were incubated on ice for 20 min, mixed with a dye solution, and loaded on polyacrylamide gels. Electrophoresis conditions for each experiment are indicated in the figure legends. Wet gels were sealed in polyethylene and exposed to x-ray film at −70°C.

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a

Mutation	Location	PCR primers (5' → 3')	PCR product size (bp)	Lesion	Mismatches formed upon reannealing of PCR product
ΔF508	exon 10	(1) CTCAGTTTTCCTGGATTATGCC (2) TGGCATGCTTTGATGACG	100	3 bp deletion (Phe deletion)	CTT + GAA bulges
		(1) CTCAGTTTTCCTGGATTATGCC (3) CTAACCGATTGAATATGGAGCC	200		
		(4) CAAGTGAATCCTGAGCGTGA (3) CTAACCGATTGAATATGGAGCC	340		
		(5) GCAGAGTACCTGAAACAGGA (6) CATTCACAGTAGCTTACCCA	491		
R553X	exon 11	(7) GCCTTTCAAATTCAGATTGAGC (9) GACATTTACAGCAAATGCTTGC	203	C → T (Arg $_{553}$ → Stop)	C/A + T/G single-base mismatches
		(10) CAACTGTGGTTAAAGCAATAGTGT (11) GCACAGATTCTGAGTAACCATAAT	425		
G551D	exon 11	(7) GCCTTTCAAATTCAGATTGAGC (9) GACATTTACAGCAAATGCTTGC	203	G → A (Gly $_{551}$ → Asp)	G/T + A/C single-base mismatches
G542X	exon 11	(7) GCCTTTCAAATTCAGATTGAGC (8) TGCTCGTTGACCTCCACTC	141	G → T (Gly $_{542}$ → Stop)	G/A + T/C single-base mismatches

b

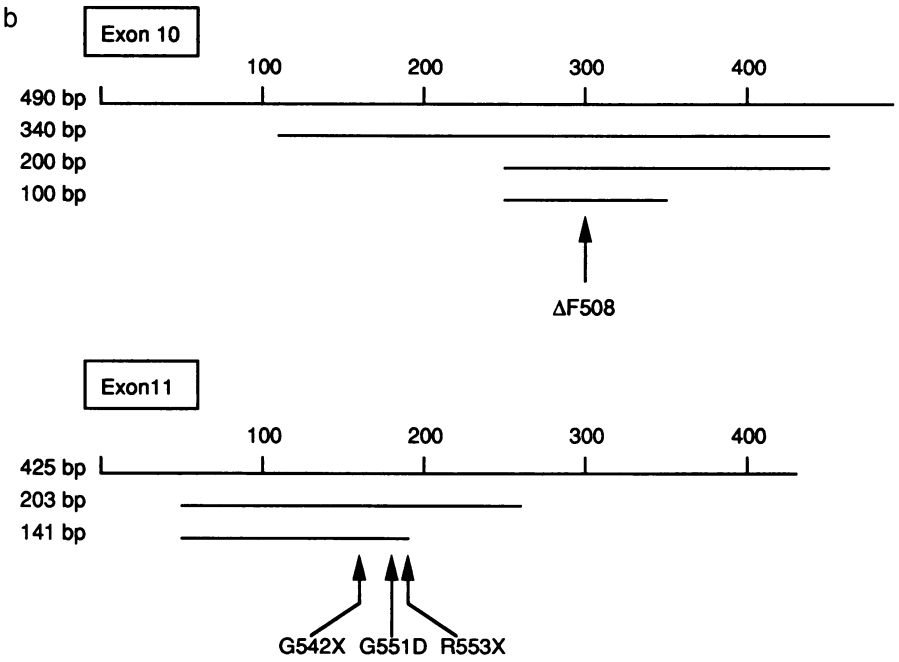

FIG. 1. (*a*) Mutations within the *CFTR* gene and respective PCR products. (*b*) Positions of *CFTR* mutations within PCR-amplified DNA fragments.

RESULTS AND DISCUSSION

Experimental Strategy. A schematic representation of our experimental design is shown in Fig. 2. A DNA region of interest is PCR amplified from genomic DNA samples extracted from peripheral blood. PCR products are heat denatured and reannealed. If the target DNA is heterozygous at the *A* locus (*A*/*a*), reannealing of a PCR product yields four different DNA species: two homoduplexes, *AA* and *aa*, and two heteroduplexes, *Aa* and *aA*. In the case of a homozygous allele only homoduplexes are generated upon reannealing of the PCR product. Depending on the nature of the difference between the two alleles, the resulting heteroduplexes contain various single base-pair mismatches or small loops in one of the strands. MutS protein binds to mismatched base pairs, and formation of the complex is detected by a gel mobility-shift assay. MutS binds to homoduplex DNA poorly and the difference in binding of heteroduplexes versus homoduplexes is the signal upon which this technique is based.

Carrier Detection of ΔF508 Mutation in the *CFTR* Gene. The carrier frequency for this severe disease is ≈1 in 25 in North American Caucasians; 1 individual in 2500 inherits two altered copies and is thus affected with the disease. The most frequent mutant allele of the *CFTR* gene, ΔF508, is a 3-bp deletion in the coding region of the gene that results in a loss of a phenylalanine residue at amino acid position 508 (Fig. 1*a*). This mutation accounts for ≈75% of *cftr* chromosomes in this population.

Genomic DNA samples extracted from peripheral blood were amplified by using the ^{32}P-5'-end-labeled primers that bracket the site of ΔF508 deletion to generate a PCR product 100 bp long. Heteroduplexes with this deletion in one of the

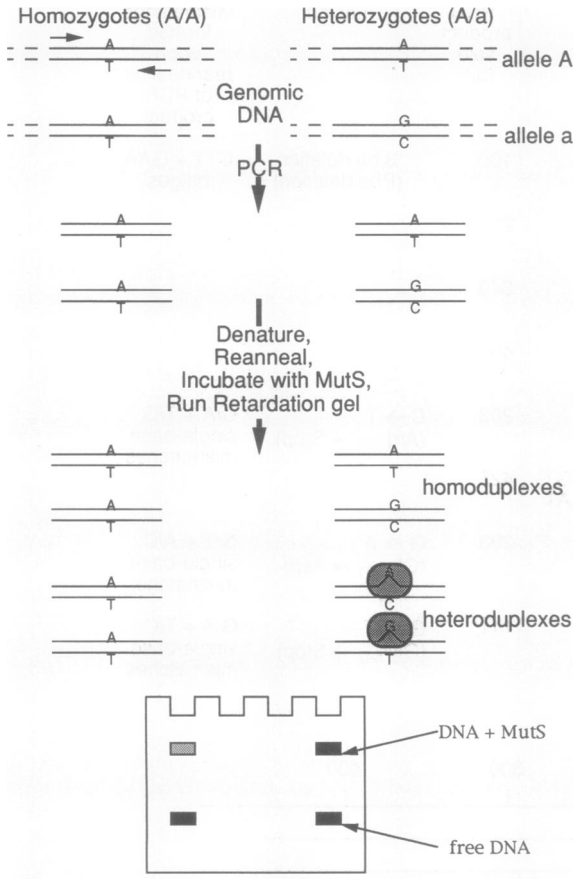

FIG. 2. Experimental strategy (schematic representation).

strands contain a CTT or a GAA bulge in the complementary strand. This structural distortion induces a kink of ≈80° in DNA molecules (9). After reannealing and incubation with MutS protein, the reaction mixture was electrophoretically

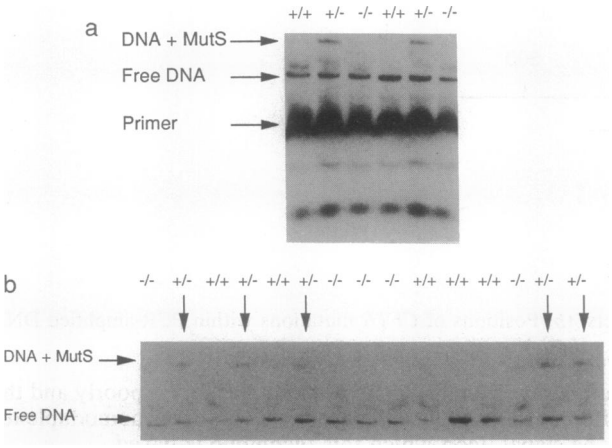

FIG. 3. Detection of heterozygosity for ΔF508 by mobility-shift assay. (*a*) A representative band-shift assay is shown. Control experiments established that the band labeled DNA + MutS depended on addition of MutS protein and hence was not due to altered mobility of heteroduplex DNA *per se*. (*b*) Detection of *CFTR* carriers among a mixed population of carriers, unaffected people, and affected people (only the essential area of the gel is shown). Genomic DNA (250 ng) was amplified with *Taq* polymerase to generate fragments 100 bp long; 27 cycles were performed. Mobility-shift assay was performed in a 6% polyacrylamide gel (20 × 20 × 0.15 cm) (AA/Bis, 19:1) (Bio-Rad) in 0.2× TBE. The gels were run at 4°C for 15 min at 500 V. +, Normal allele; −, mutant allele; +/+, homozygous healthy individuals; −/−, homozygous affected patients; +/−, asymptomatic heterozygous carriers.

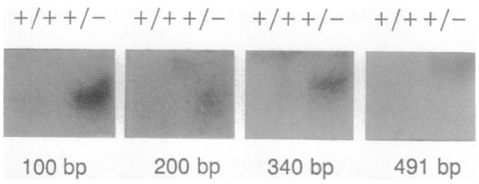

FIG. 4. Effect of PCR product size on ΔF508 detection. Genomic DNA was amplified with *Pfu* polymerase; 27 cycles were performed. The mobility-shift assay was performed in a 4–15% precast gradient minigel (Bio-Rad) in the discontinuous Laemmli buffer system (10) without SDS. The gel was run at 200 V for 30 min (100 bp) and for 45 min (200, 340, and 491 bp). Only the area of the gel containing shifted bands is shown.

separated on a 6% polyacrylamide gel (Fig. 3*a*). MutS binding, detected as a shifted band, was consistently stronger in the amplified DNA of individuals who were heterozygous for ΔF508 than in the amplified DNA of homozygotes (both affected and normal individuals). The mobility shift caused by MutS protein binding DNA is much larger than a slight gel retardation exhibited by molecules of heteroduplex DNA (data not shown). In a blind experiment, all 5 heterozygous carriers of the ΔF508 mutation were correctly detected among 15 individuals (Fig. 3*b*).

One of the problems we encountered in the course of this study was the low stability of MutS–DNA complexes, resulting in their dissociation and, after long electrophoretic runs, in loss of signal. On the other hand, the wavy bands, which represent DNA dissociated from the complex with MutS, were seen above free DNA in all the lanes in Fig. 3, suggesting that MutS protein initially bound not only heteroduplexes but homoduplexes as well. Apparently, the latter nonspecific complexes had a shorter half-life than complexes of MutS bound to mismatched bases. Therefore, the choice of the duration of a run was a compromise between these two considerations and was rather short (15 min in Fig. 3). Omission of EDTA from 0.2× TBE buffer did not appear to improve the stability of the complexes. In the discontinuous Laemmli buffer system (10) used in the experiments in Figs. 4 and 5, the MutS–DNA complexes remained stable for a longer time (30–45 min), but shifted bands appeared as smudges, revealing inherent heterogeneity of the complexes. Stability of the complexes also appeared to increase with DNA length.

Heterozygosity for ΔF508 is easily detectable by a variety of different methods (11, 12). However, due to its pronounced structural aberration and its high allele frequency, we used this mutation to examine certain experimental parameters that may influence MutS protein performance in a band-shift assay.

Parameters That Affect the Ability of MutS to Detect Heterozygosity for ΔF508. *A priori*, the main caveat in using MutS protein to evaluate mutations in PCR-amplified DNA was a low fidelity

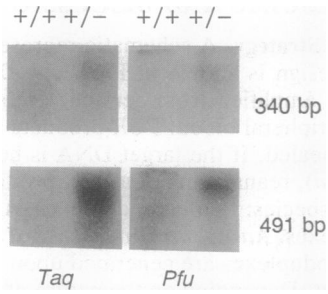

FIG. 5. Effect of polymerase fidelity on ΔF508 detection. Genomic DNA was amplified with *Taq* or *Pfu* polymerase; 27 cycles were performed. Electrophoresis conditions were as in Fig. 4, except the gels were run for 30 min (340 bp) and 40 min (491 bp).

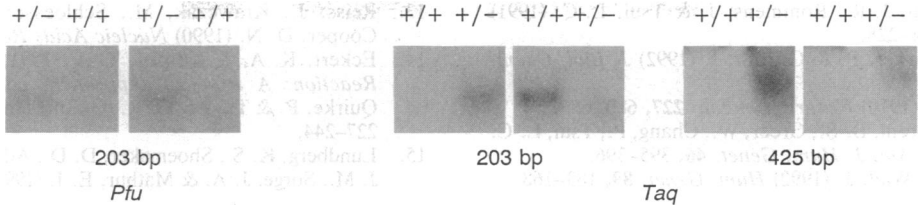

FIG. 6. Detection of heterozygosity for R553X point mutation. Genomic DNA was amplified with *Pfu* or *Taq* polymerase; 27 cycles were performed. Electrophoresis conditions were as in Fig. 4, except that the gel was run for 30 min. Similar results were obtained with G542X and G551D, as described in Fig. 1*a* (data not shown).

of thermostable DNA polymerases. Upon reannealing of amplified DNA, any polymerase errors that accumulated during amplification would result in artificially introduced mismatches. MutS protein binding to such erroneous mismatches could account for the noise in our experiments (appearance of shifted bands in lanes −/− or +/+). A noise level significantly lower than the signal produced by binding of MutS protein to mismatches originating from heterozygosity (shifted bands in lanes +/−) would still be acceptable. The number of error-caused mismatches would increase with the length of PCR product and depend on the fidelity of the polymerase used for amplification (13). Therefore, we examined the influence of these two factors on the signal/noise ratio in our mobility-shift experiments.

Effect of PCR Product Size. To test the ability of MutS protein to select 3-bp bulges in different-sized DNA molecules, 3 more pairs of primers were synthesized. The resulting PCR products and relative positions of the ΔF508 deletion within them are shown in Fig. 1*b*. The data in Fig. 4 illustrate a successful detection of heterozygosity for ΔF508 using PCR products 100, 200, 340, and 491 bp long. There were no reliable differences in the results with different-sized PCR products to indicate any optimum size for these experiments (see also Figs. 5 and 6).

Effect of Polymerase Fidelity. *Taq* DNA polymerase lacks 3′ → 5′ proofreading exonuclease activity and, therefore, exhibits a higher misincorporation rate than thermolabile DNA polymerases (14). The fidelity of *Pfu* DNA polymerase is reported to be 12-fold higher than the fidelity of *Taq* polymerase, and *Pfu* DNA polymerase has the highest known fidelity among thermostable DNA polymerases (15). For the data presented in Fig. 4, amplification was carried out with *Pfu* polymerase. The results of mobility-shift assays performed on reannealed PCR products 340 and 491 bp long, each amplified with *Taq* and *Pfu* polymerase, respectively, are compared in Fig. 5. The signal/noise ratio (lanes +/− vs. +/+) appears to be adequate for PCR products amplified by using either polymerase, with *Pfu* polymerase performing no better than *Taq* polymerase under these conditions.

Detection of Heterozygous Point Mutations. As pointed out above, the ΔF508 deletion generates kinked heteroduplexes that display reduced electrophoretic mobility. Our preliminary mobility-shift experiments using synthetic double-stranded oligonucleotides indicated that MutS protein bound more strongly to mismatched heteroduplexes containing a 3-bp deletion in one of the strands than to heteroduplexes with a single base-pair mismatch (data not shown). Therefore, successful detection of point mutations with this technique utilizing PCR-amplified DNA was less certain than in the case of ΔF508. To test the ability of this method to detect point mutations, we tested the most frequently occurring non-ΔF508 mutations in CFTR, which are found in exon 11 (Fig. 1). The results obtained for the R553X C → T transition are shown in Fig. 6. Reannealed PCR products contained G/A and A/C mismatches. MutS was capable of distinguishing between heterozygotes and homozygotes for both 203- and 425-bp PCR products amplified with either *Pfu* or *Taq* polymerase. Similar results were also obtained for the G542X and G551D point mutations using 141- and 203-bp PCR products, respectively (data not shown). Since detection of point mutations using single-stranded conformational polymorphism, denaturing gradient gel electrophoresis, or mutation detection enhancement gels requires relatively long or complicated electrophoretic runs (1); the band shifts with MutS protein may offer higher through-put in the future.

CONCLUSIONS

In this paper, we presented a PCR-based technique for detecting mutations in genomic DNA by using a mismatch binding protein, MutS. This technique made use of the preferential affinity of MutS protein for DNA heteroduplexes containing mismatches caused by mutations. The binding was detected by a band-shift assay. This approach was tested on a 3-bp deletion and several single-base substitutions within the *CFTR* gene using PCR products up to ≈500 bp long. MutS protein binds different mismatches with different affinities, and some mismatches (e.g., A/C) are, in fact, bound only poorly (7). Each pair of alleles produces two different mismatches in this protocol. Thus, although we don't know whether both species of heteroduplexes are bound equally in these experiments, the existence of two species from each heterozygote may increase the reliability of the method. This method appears to have a potential for diagnostic applications. In its current version, it does not differentiate between homozygotes with both wild-type or both mutant alleles, but it can be modified to do so by reannealing PCR products with an excess of a probe corresponding to one of the strands of the wild-type allele. The ratio of the signals after MutS binding would be 0:1:2 for +/+, +/−, and −/− DNA, respectively. Preliminary experiments indicate that MutS protein immobilized onto a solid support retains its binding activity and specificity. Thus, it may be possible to develop a non-gel format of the technique described here, which would offer a greater potential for automation.

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