MUTATION DETECTION BY CLEAVASE® FRAGMENT LENGTH POLYMORPHISM ANALYSIS

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ABSTRACT

We describe a new method for mutation detection based on cleavage patterns generated by a structure-specific thermostable endonuclease, CLEAVASE® I. The analysis of 1,059-bp DNA fragments derived from wild-type and mutant forms of the human tyrosinase gene and differing by a single base change could be distinguished by changes in the band pattern and intensity. Mutations were localized near their known region on the DNA fragment. Differential labeling of each DNA strand allowed both strands to be analyzed simultaneously. The method of CLEAVASE Fragment Length Polymorphism (CFLP™) is simple, rapid, and cost effective compared to other mutation detection techniques.

Methods for detecting and characterizing nucleotide sequence changes have become crucial for the analysis of loci important in human cancer and genetic disease, the rapid assessment of drug resistance in bacterial pathogens, and the genetic typing of genes. The demand for rapid and easy tests to characterize unknown sequences has increased.

The current techniques vary in their complexity, technical skill level required, and detail of data obtained. A DNA fragment may be characterized by several methods yielding information ranging from the size of the fragment to the DNA sequence. Single strand conformation polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE) analysis are technically demanding and are limited to analysis of fragments no longer than ~250 bp or 600 bp, respectively (1,2). Neither method gives information about the location of variations detected. Alternatively, DNA sequence analysis is time consuming and limited to analysis of DNA fragments of 500 bp or less per assay.

Following denaturation, DNA strands assume folded hairpin-like structures that are unique to the nucleotide sequence of the molecule (3). This observation formed the basis for the development of SSCP. We have identified an endonuclease, CLEAVASE I, that recognizes and cleaves these folded structures at the junctions between the single-stranded and duplexed regions. Using this enzyme, we developed a method to characterize single base differences in DNA molecules. For the CFLP reaction, the 5′ ends of DNA fragments generated by PCR are differentially labeled with either biotin, fluorescein, or JOE; heat denatured; and cooled to a preoptimized temperature. CLEAVASE I is added, the reaction proceeds for 2 to 5 min, and the reaction is stopped with a formamide dye mixture. Following rapid electrophoretic resolution and visualization of the cleavage products on denaturing polyacrylamide gels, a unique, reproducible pattern or bar code is generated (figure 1). The resolution of cleavage products by rapid electrophoresis through short denaturing polyacrylamide gels allows the analysis of large DNA fragments. In addition to revealing sequence differences between two DNA fragments, the patterns often reflect the locations of the sequence differences.

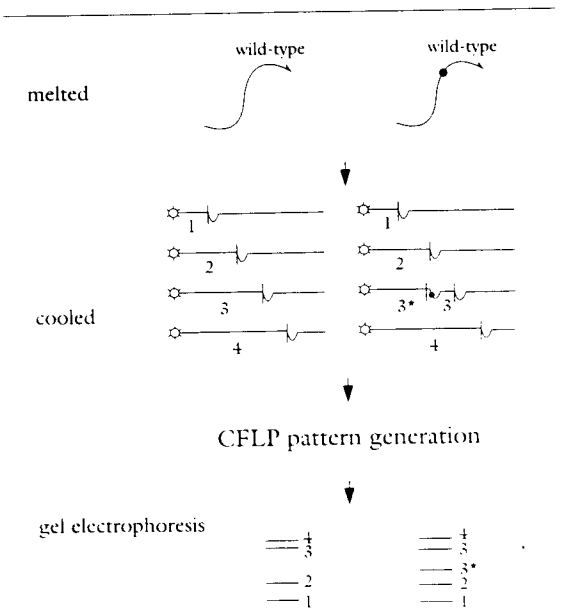

FIGURE 1. Schematic of the CFLP reaction.

METHODS

Plasmid templates. Plasmids containing the wild-type and two mutant alleles, R422Q and G419R, of the human tyrosinase gene were used to generate PCR products (157 bp and 1,059 bp) and were the gift of Dr. Richard Spritz, University of Wisconsin, Madison (4). The three primers used were B, Exon4tyr CAC CGT CCT CTT CAA GAA G; F, Exon4tyr CTG AAT CTT GTA GAT AGC TA; and Exon1tyr, GCA ATT TGG CTT TTG GGG A. The primers were labeled at their 5′ ends with either biotin, fluorescein, or JOE to generate PCR fragments labeled at either one or both 5′ ends.

TABLE 1. Components of the CFLP Power Scan System.

Component	Amount
CLEAVASE I Enzyme (25 units/µl)	100 µl
10X CFLP Buffer	250 µl
2 mM $MnCl_2$	250 µl
10 mM $MgCl_2$	250 µl
Stop Solution	1.6 ml
Distilled Water	2 × 1.0 ml
Wild-Type Control DNA	10 µl
Mutant Control DNA	10 µl

PCR. PCR contained 1 to 5 ng of template DNA, 2.5 units recombinant *Taq* DNA polymerase, 25 pmoles of each primer, 1X PCR buffer [20 mM Tris-HCl (pH 8.5), 1.5 mM $MgCl_2$, 50 mM KCl, 0.5% Tween® 20, and 0.5% Nonidet® P-40], and 50 µM of each dNTP in a final volume of 100 µl. Amplification was 35 cycles with denaturation at 94°C and extension at 72°C for 5 min. Annealing temperatures were 50°C for the 157-bp fragment (exon 4) and 52°C for the 1,059-bp fragment (exons 1–4). PCR products were purified by elution from denaturing polyacrylamide gels (5).

CFLP reactions. The CFLP reactions were performed using the CFLP POWERSCAN™ System (Cat. No. 10647, table 1). For DNA fragments <500 bp, ~100 fmoles of the DNA fragment labeled at one of the 5′ ends was combined with water in a final volume of 15 µl. An

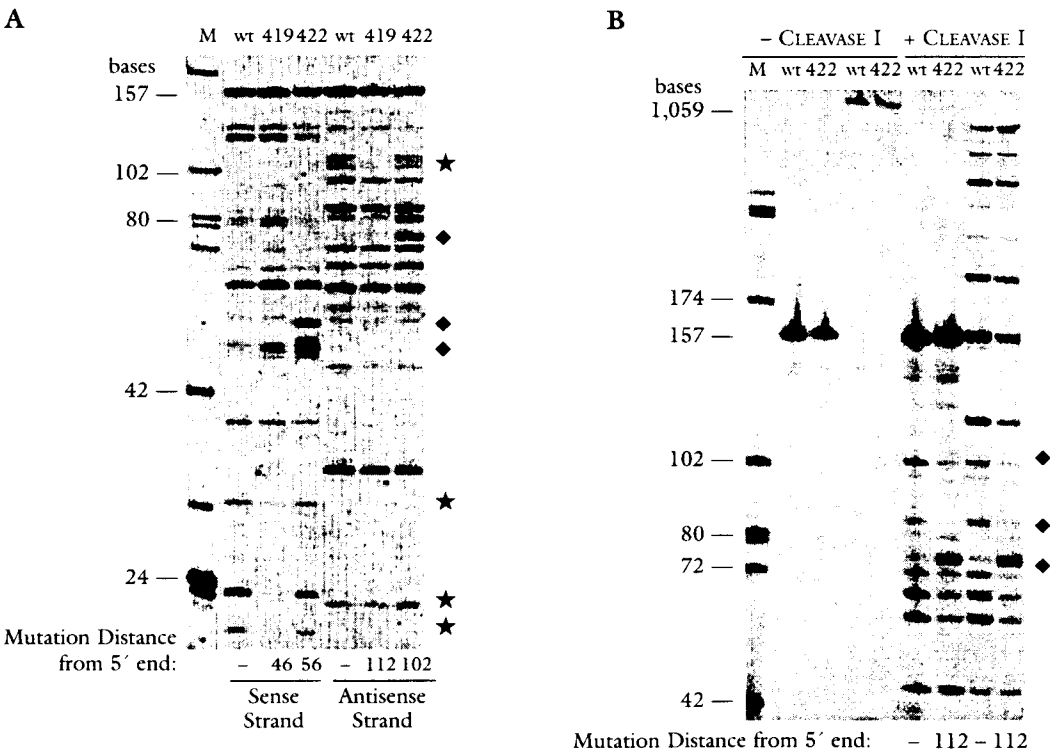

FIGURE 2. CFLP analysis of DNA fragments. Panel A. Analysis of both strands of a 157-bp fragment. Mutants G419R and R422Q, and wild-type amplicons containing 5′ biotinylated sense strands and 5′ fluoresceinated antisense strands were subjected to CFLP analysis. A 10% polyacrylamide gel was electrophoresed for 15 min. Lane M is the DNA size standards. Mutational changes are marked with ★ for G419R and ◆ for R422Q. Panel B. Mutation localization in different-sized fragments. Biotin-labeled amplicons of 157 bp and 1,059 bp derived from wild-type and mutant R422Q genes are shown both uncut and following CFLP analysis. A 6% polyacrylamide gel was electrophoresed for 30 min. Lane M is the DNA size standard.

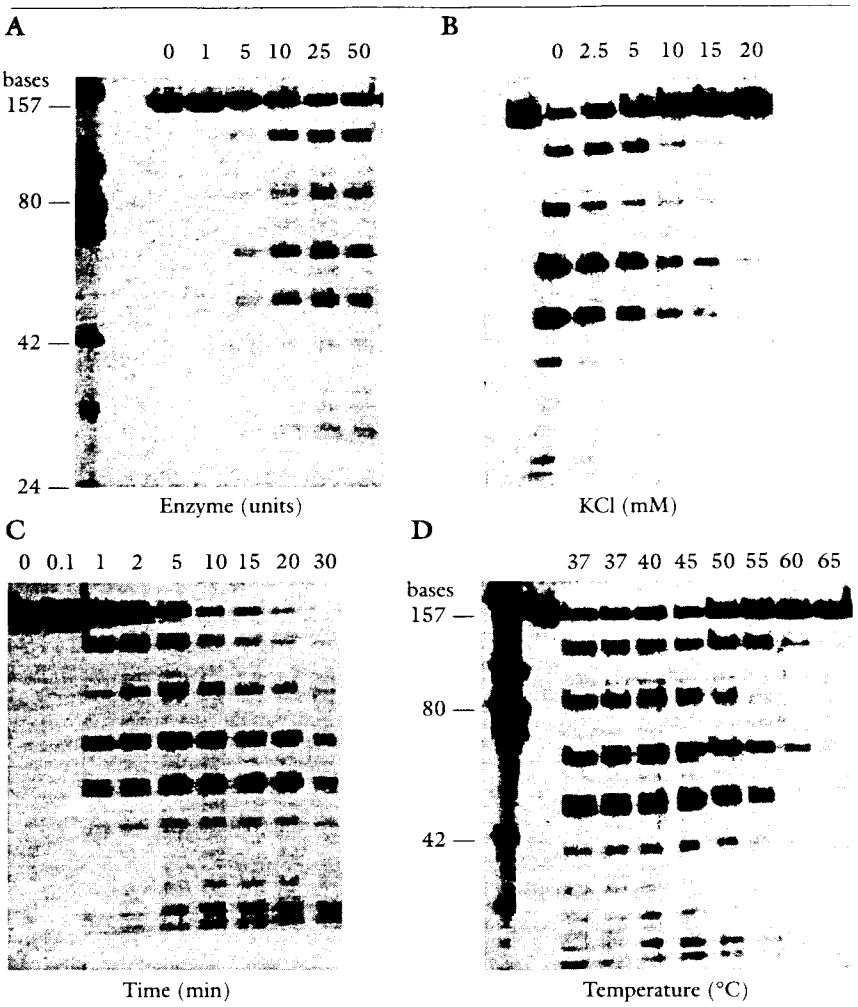

FIGURE 3. CFLP reaction parameters. A biotinylated 157-bp wild-type tyrosinase amplicon was used in CFLP with varying concentrations of the CLEAVASE I enzyme (Panel A) and KCl (Panel B), reaction times (Panel C), and temperatures (Panel D). CFLP reactions were for 2 min using 25 units of enzyme at 45°C except where otherwise indicated. The gels were 6% polyacrylamide.

enzyme mixture containing 2 µl of 10X CFLP buffer [100 mM MOPS (pH 7.5), 0.5% Tween 20, 0.5% Nonidet P-40], 2 µl of 2 mM $MnCl_2$, and 25 units of CLEAVASE I (1 µl) was prepared. The DNA was heated to 95°C for 15 s and cooled to the optimal reaction temperature. The optimal temperatures were 45°C for the 157-bp fragment and 55° for the 1,059-bp fragment. As soon as the DNA cooled to the optimal temperature, 5 µl of enzyme mixture were added and incubated for 2 to 5 min. 16 µl of stop solution [95% formamide, 10 mM EDTA (pH 8.0), 0.05% xylene cyanol, 0.05% bromophenol blue] were added. The CFLP patterns were resolved by electrophoresis through small (10 cm × 10 cm × 0.5 mm or 20 cm × 20 cm × 0.5 cm) 6% or 10% denaturing polyacrylamide gels. For DNA fragments >500 bp or for fragments that showed consistent overdigestion, the final CFLP reaction mixtures also contained 1 mM $MgCl_2$.

Detection of CFLP patterns. For DNA fragments labeled with biotin, the DNA was transferred to a positively charged nylon membrane by the method of Southern (6). A streptavidin-alkaline phosphatase conjugate was added. For fluorescein-labeled DNA, the DNA was transferred as described above and an alkaline phosphatase anti-fluorescein IgG conjugate was added. Labeled DNAs were detected with a chemiluminescent substrate (CDP-Star™) and exposure to X-ray film.

RESULTS

Detection and localization of single base mutations. Both strands of a 157-bp region of the wild-type and mutant alleles of the tyrosinase gene were analyzed by CFLP (figure 2). Comparison of the patterns generated from the wild-type and mutant fragments revealed changes that correlated with the position of each mutation on either strand. On the sense strand, the G419R mutation (mutation at 46 nucleotides from the 5′ end) resulted in multiple changes such that the majority of bands <40 nucleotides were missing. In the R422Q mutation (mutation of 56 nucleotides), a single band migrating at ~60 nucleotides was shifted downward and was more pronounced as well as several changes at 50 nucleotides. Similar changes were observed on the antisense strand, but the changes were shifted farther up the gel, reflecting the greater distance between the mutations and the 5′-labeled end on this strand. The G419R mutation on the antisense strand showed a decreased intensity in a group of bands migrating ~100 nucleotides, whereas the R422Q mutation resulted in the appearance of a strong band migrating at 80 nucleotides. The changes observed for both strands of the G419R and R422Q amplicons mapped to within 40 nucleotides of the position of the actual known mutation.

It appeared that the band changes generally occurred in the region of the mutation. The close agreement of the localization of the mutations in each strand suggested that CLEAVASE I detected perturbations of the local single-stranded secondary structure rather than long-range intrastrand interactions between distal portions of the DNA. To test this hypothesis,

amplicons derived from wild-type and R422Q mutant were created that contained the same 5′ end but extended either 157 or 1,059 nucleotides. The mutation was 102 nucleotides from the 5′ end of the antisense strands. Analysis of the CFLP banding patterns of the antisense strands were similar <100 nucleotides (figure 2B). The R422Q mutation appeared as an intense, slightly shifted band at 80 nucleotides in both the fragments, and the band at ~100 nucleotides disappeared. (The change at ~100 nucleotides was obscured in figure 2A due to different electrophoresis conditions.) The additional 900 bp did not substantially alter the CFLP pattern in the 5′ portion of the 1,059-bp fragment, suggesting that the conditions for CFLP analysis resulted primarily in the formation of local intrastrand structures.

CFLP reaction conditions. Factors that affect the CFLP reactions were examined (figure 3). The CFLP reaction showed broad ranges of tolerance for the concentration of enzyme (10 to 50 units), time of incubation (5 to 15 min), and reaction temperature (37 to 50°C). However, salt had a marked effect even at low concentrations. The activity of CLEAVASE I is considerably lower in high-salt conditions (data not shown).

In some cases, longer DNA fragments were extremely susceptible to overdigestion. In the presence of 0.2 mM $MnCl_2$ and 1 mM $MgCl_2$, the CFLP reaction kinetics were reduced 3- to 4-fold, thus preserving the complete CFLP pattern (data not shown).

DISCUSSION

We have described a new mutation detection technique with the capability of detecting single base changes and localizing the changes to the relevant genetic region. A single point mutation in a 1,059-bp fragment was detected, and we have successfully analyzed fragments as long as 1,600 bp in a single reaction (data not shown). Both strands can be analyzed simultaneously, allowing confirmation of the data. The reproducibility of the CFLP reaction will allow the generation of genetic bar code libraries characteristic of particular normal and mutant genetic alleles. Also, incorporation of dUTP did not interfere with the detection of mutations (data not shown), making the CFLP reaction amenable to routine laboratory usage and decontamination with uracil DNA glycosylase (UDG). However, DNA containing dUTP can only be compared to other fragments containing dUTP.

The CFLP technique should find wide utility for mutation detection in a number of different systems. Other systems we have examined include alleles of the p53, β-globin, MSH-2, and tyrosinase genes with a 100% rate of mutation detection, as well as genotyping hepatitis C and detecting point mutations associated with rifampin and isoniazid drug resistant phenotypes of *Mycobacterium tuberculosis.* Finally, CFLP has been used to differentiate bacterial species by analyzing amplicons generated by amplification of the bacterial 16S rRNA genes of *Escherichia coli*, including 0157:H7 serotypes, *Salmonella typhi*, *Shigella dysenteriae*, *Campylobacter jejuni*, *Staphylococcus aureus*, and *Staphylococcus epidermitis.* The use of CLEAVASE I for mutation detection is as simple as the use of a restriction endonuclease and allows rapid, reproducible molecular mutation detection.

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