Nonradioisotopic PCR
Heteroduplex Analysis:
A Rapid, Reliable Method of Detecting Minor Gene Mutations


Gene mutations play an important role in the molecular basis of diseases including cancer. In addition to sequencing, there are several methods to detect minor mutations, even single-base changes (1,3). However, none of these detect all of the minor molecular changes that can occur. White et al. (3) used $^{32}$P-labeled heteroduplex polymorphism to visualize single-base substitutions. We have modified their method by making use of ethidium bromide fluorescence to detect re-annealed heteroduplexes. The method requires only small amounts of polymerase chain reaction (PCR)-amplified DNA, is easy to handle because radioactivity is not involved and readily resolves differences between mutant and control DNA as we report here.

The gpt gene in PL61/1 cells was mutatred by $\gamma$-rays, and mutants were selected by resistance to 6-thioguanine (6-TG) challenge (2,4). DNA from control- and mutant-cell lysates were separately amplified by using PCR using the same pair of primers (2). From lysates, these primers yielded a 561-bp fragment of the gpt gene. The PCR amplification was verified by DNA electrophoresis in a gel of 1% agarose in 1× TBE buffer (0.095 M Tris-borate, 0.002 M EDTA, pH 8.0) containing 0.5 $\mu$g/mL ethidium bromide. For heteroduplex analysis (HDA), 3.0 $\mu$L of the PCR products from both control and mutant cells were mixed and cut with 6.0 U of KpnI restriction endonuclease. This cutting resulted in 395- and 166-bp fragments of double-stranded DNA in the normal gpt gene. Denaturations and renaturations were performed in a programmed PCR thermal cycler (Perkin-Elmer, Norwalk, CT, USA); 95°C for 8 min, followed by 1 min at 90°C, 1 min at 80°C, 1 min at 70°C and so on until room temperature was reached. For polyacrylamide gel electrophoresis (PAGE), the renatured sam-

Figure 1. Examples of the sensitivity and resolution of the method in the analysis of heteroduplexes in a 10% non-denaturing PAGE with 10% glycerol. Bands were visualized by ethidium bromide fluorescence. Lanes 1–8 were from PCR-amplified DNA samples of the gpt gene from 6-thioguanine-resistant mutants, which had been cut with KpnI. Lane C was from DNA from a normal control treated in the same way, and lane M was a 1-kb DNA ladder for a size marker.
samples were loaded, along with 2.0 µL of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol), into a 1.5-mm thick, 20-cm × 20-cm square, 10% non-denaturing polyacrylamide gels containing 10% glycerol and 0.5% agarose in 1× TBE buffer. The buffer was recirculated during electrophoresis, which was run at 200 V, 23 mA for 10 min; then 180 V, 19 mA for 1 h followed by 140 V, 17 mA for 22 h—all at 4°C. After electrophoresis, the gels were stained with 0.5 µg/mL of ethidium bromide in 1× TBE buffer for 40–60 min. DNA bands were observed with ultraviolet (UV) light.

Typically, four kinds of DNA duplexes were formed from the reassociation of the denatured products: two sizes of homoduplexes from control and mutant DNA duplexes; and two kinds of electrophoretically distinct heteroduplexes from the cross-annelings between control and mutant strands. Lane 3 in Figure 1 is an example of the four kinds of duplexes formed from the longer restricted fragments of control and mutant DNA. The two homoduplexes between DNA markers 344 and 396 bp are approximately 12 bp apart as estimated densitometrically with a Digital Imaging System (Model IS1000; Alpha Innotech, San Leandro, CA, USA) and a DNA ladder, lane M. Usually the differences in the homoduplexes were too small to be seen with PAGE. However, the heteroduplexes give rise to reduced mobilities and distinctive, separated bands, e.g., lanes 1, 4, 6 and 7. In view of the sensitivity of the method, it appeared that the mutant DNA samples used in lanes 1, 4 and 7 had similar mutations.

The usefulness of HDA to detect minor mutations was made evident by a comparison with ethidium bromide staining and single-stranded conformational polymorphism (SSCP) (4). To begin with, SSCP was found to be optimally sensitive over a smaller size range than HDA; with the latter method, the range is around 400 bp or longer. Without cutting, HDA of the 561-bp PCR fragment of gpt was found to be slightly less sensitive than duplex DNA of approximately 400 bp (4). The sensitivities of both methods were about the same for the 395-bp fragment (i.e., mutants were detected in 90% of the samples). But the HDA analysis showed larger, more easily identified differences between the control and mutant DNA. Nonradioisotopic, ethidium bromide visualization in HDA was found to be quite effective in detecting differences of around 400 bp, even though small samples, 3 µL, were used as in Figure 1. The sample size used was similar to that in the method of White et al. (3), but, in contrast, 32P-dCTP was not needed. PAGE with 1.5-mm gels facilitated the detection by heteroduplex formation mutants that could not be distinguished by conventional electrophoresis in 1% agarose (data not shown). The faint heteroduplex bands in lane 8 apparently were caused by a deletion in the 166-bp fragment (note the greater mobility of this fragment). The faintness was probably due to less ethidium bromide being bound to the smaller strands. Lane 2 was from a sample with a large deletion (ca. 200 bp) including the restriction site, which prevented the formation of heteroduplexes. Lane 5 was from a mutant, which was not detected by heteroduplex formation.

Nonradioisotopic HDA is a straightforward method and should be useful in both clinical diagnosis and basic research for the rapid screening of small molecular changes that cannot be distinguished in conventional agarose gel electrophoresis or even PAGE. At the very least, it can complement other techniques. Sequencing can be used when “hot spots” as “signatures” are detected compared with known mutants.

REFERENCES

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Single-Tube Immuno-Capture and PCR of Genotoxin-Modified DNA: Application to Gene-Specific Damage Analysis


Growing interest in gene-specific repair has stimulated a search for sensitive methods to quantitatively assess the induction and repair of genotoxic DNA adducts in individual genes (1). We have recently described an immuno-polymerase chain reaction (PCR)-based technique, which combines the sensitivity of antibodies with the power of gene amplification (3). While other PCR-based methods make use of the decrease in the amount of PCR signal from the modified template for quantitation of damage (4,5), our assay assumes a proportionality between the