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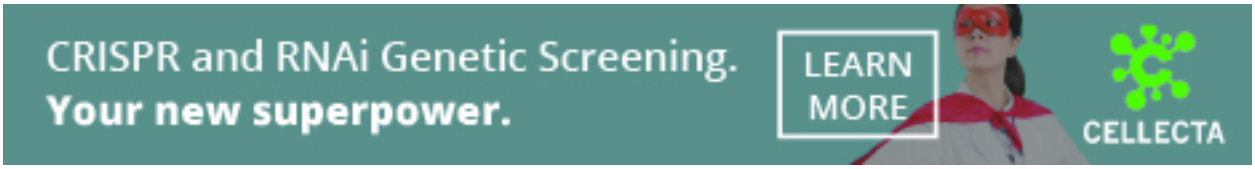

Sensitivity of Detection of Heterozygous Point Mutations in p53 cDNAs by Direct PCR Sequencing

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One efficient way of identifying point mutations in specific genes is by direct sequencing of PCR-amplified cDNA. In a previous study,[1] we found that not all existing heterozygous mutations in the p53 gene were readily detectable on DNA sequencing gels in each independent repeat experiment. To determine the sensitivity of detection of point mutations in cDNAs in the presence of wild-type cDNA sequences on DNA sequencing gels, we performed a quantitative sequencing study by mixing wild-type p53 cDNA with mutant p53 cDNA at different ratios. The results show that in mixtures of mutant and wild-type cDNA sequences the mutant allele could be detected by direct sequencing of PCR-amplified cDNA as long as it represented 20% or more of the allelic mixture.

RESULTS AND DISCUSSION

The identification of point mutations in human genes has contributed greatly to our understanding of the molecular mechanism of human diseases. Various methods have been developed for the screening of mutations in mammalian genomic DNA or cDNA. The most commonly used techniques for the identification of known mutations include restriction fragment length polymorphism (RFLP) analysis and mutation-specific oligonucleotide hybridization.[2] For the detection of unknown mutations, ribonuclease cleavage of mismatches between a labeled normal antisense RNA and genomic DNA as well as cleavage of RNA–RNA duplexes have been successfully used.[3,4] Mutations present in genomic DNA have also been studied by the use of denaturing gel electrophoresis,[5,6] the chemical modification and cleavage method,[7] and single-strand conformation polymorphism (SSCP) analysis.[8]

PCR amplification of specific segments of DNA implicated in frequently mutated genes also constitutes a powerful tool for the identification and characterization of mutations. Small rearrangements or deletions that may go undetected in Southern blot analyses can be detected using amplification of subregions of cDNA and subsequent migration patterns on agarose gels. Point mutations can be detected by sequencing single-stranded cDNA produced by asymmetric PCR reactions, using unequal molar ratios of amplification primers. Direct sequencing of this single-stranded template yields reproducible, high-quality sequence data.[9] An important advantage of this sequencing method is that it facilitates the simultaneous detection of alleles present in a heterozygous state.[1,9] In contrast, detection and sequencing of heterozygous alleles via the cDNA cloning procedure requires the subcloning of at least several cDNA clones, followed by the complete sequencing of each of the clones. Furthermore, in an aneuploid cell, the gene to be sequenced may be present in more than two copies (e.g., four copies of the p53 gene on chromosome 17p in the Jurkat cell line; see ref. 1), requiring the independent isolation and sequencing of a large number of cDNA clones. The cloning method is further problematic when PCR-amplified DNA is used in the cloning procedure because the probability of inducing nucleotide misincorporation is high, while misincorporation does not interfere with the direct sequencing procedure.

The failure to detect existing point mutations may occur when tumor tissue comes mixed with a large concentration of normal tissue, or in cells or cell lines in which mutated alleles are expressed at a much lower level than wild-type alleles, or vice versa. This phenomenon was evident in mapping experiments of heterozygous p53 gene mutations in human leukemia cell lines,[1] and was likely due to differential expression of the alleles.

The problem of reproducible detection of mixed allele sequencing was addressed in the current quantitative reconstruction experiment, which was designed to test the limit of detection of point mutations on a background of wild-type alleles. Mixtures of wild-type and mutant p53 cDNAs were prepared at ratios 1:1 through 6:1. The cDNA mixtures containing a total of 10 ng cDNA each were amplified by asymmetric PCR and were directly sequenced. Figure 1 shows that when wild-type p53 cDNA is mixed with mutant p53 cDNA at ratios of 1:1, 2:1, and 3:1, the mutant allele is readily detectable on the sequencing gels. At a ratio of 4:1, detection of the minority

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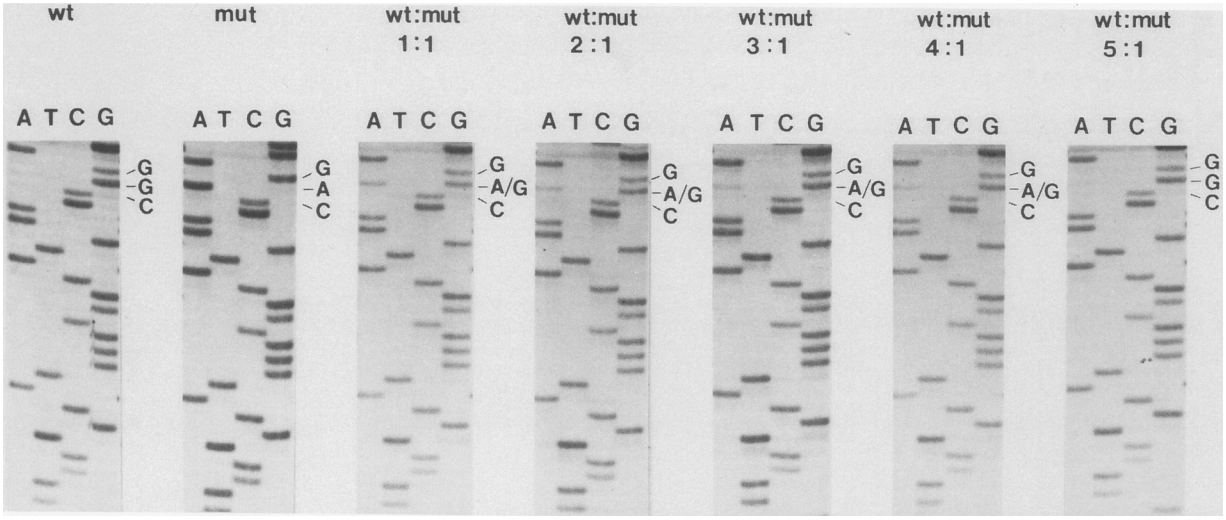

FIGURE 1 Quantitative PCR–sequencing reconstruction experiment with mixtures of wild-type and mutant p53 cDNA templates. A total of 10 ng of wild-type and mutant cDNAs were mixed at the indicated ratios, and asymmetric PCR/direct sequencing was performed. When wild-type p53 cDNA is mixed with mutant p53 cDNA at ratios 1:1, 2:1, and 3:1, the mutant allele is readily detectable. At a ratio of 4:1, detection of the mutant allele becomes ambiguous. At a ratio >5:1, the mutant allele is undetectable. Wild-type and mutant p53 cDNA inserts were isolated from p53 cDNA clones php53BAM and php53BAM-248. The wild-type construct php53BAM consists of a human wild-type p53 cDNA clone inserted into plasmid pBR322 at the *Bam*HI site.[10] php53BAM-248 is an identical construct except that a point mutation at p53 codon 248 (CGG→CAG) has been introduced. The wild-type and mutant 2.1-kb p53 cDNA inserts were cut out with the restriction enzyme *Bam*HI, and purified from a 1% agarose gel. Asymmetric PCR amplification reactions were carried out with 10 ng of the mixed 2.1-kb p53 cDNA inserts as templates, using 50 pmoles of PCR primer #6 and 1 pmole of primer #3[1] to generate 609-bp single-stranded DNA fragments. Amplification conditions were as previously described.[1] The amplified products were precipitated with 2-propanol and subjected to sequencing analysis using the dideoxy chain-termination method (USB sequencing kit).

allele becomes ambiguous, while at a ratio of 5:1 the minority allele becomes undetectable. Therefore, by using the PCR and sequencing conditions described, sequencing of mixtures of templates is feasible provided the minority template represents at least 20% of the template mixture.

These experiments yield an estimate of the sensitivity of sequencing mixed allelic samples. It appears to be unlikely that the sensitivity of the assay can be increased by a major factor by refining the sequencing technique. Thus, although the PCR/direct sequencing methodology has a finite sensitivity, the feasibility of simultaneously detecting and sequencing heterozygous mutations may make this approach the method of choice in many situations.

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