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F-SSCP: Fluorescence-based Polymerase Chain Reaction–Single-strand Conformation Polymorphism (PCR-SSCP) Analysis

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A fluorescence-based method for polymerase chain reaction–single-strand conformation polymorphism (PCR-SSCP) analysis, F-SSCP, was developed in which the target sequence is amplified by the PCR using fluorescent primers. The amplified products are then heat-denatured and applied to a water-jacket controlled gel in an automated DNA sequencer. The separated strands are detected as laser-excited fluorescence at the bottom of the gel, and mutations are detected as shifts in the position of the peaks in the fluorogram. The system does not involve radioactivity, and the conditions of electrophoresis are more strictly controlled than in the previous system, which relied on ambient air-cooling to maintain the gel at a constant temperature. The nature of the output data allows direct quantitative interpretation, and so the relative abundance of each allele in a mixture of two or more alleles can easily be estimated. The application of F-SSCP for detection of mutations and loss of heterozygosities of p53 in tumor tissues is reported.

Polymerase chain reaction–single-strand conformation polymorphism analysis (PCR-SSCP) is a rapid and efficient method for the detection of mutations and polymorphisms in genomic or cDNA sequences.(1) In the original method, the target sequence is amplified and radioisotopically labeled simultaneously and then separated by polyacrylamide gel electrophoresis in a single-stranded state.(2) Mutations are detected as mobility shifts caused by a change in the conformation of single-stranded amplification products. Potential uses for this method in clinical DNA diagnosis include detection of germ-line mutations of various genes responsible for genetic diseases and detection of somatic mutations of oncogenes/anti-oncogenes in cancerous tissues.(1) Use of a radioisotope is, however, a disadvantage, especially in clinical tests or in analyses of large numbers of samples. Several groups have demonstrated that SSCP analysis can be performed by electrophoresis in small gels with detection of the bands by silver-staining.(3–5) These nonradioactive methods are convenient for detecting some mutations but are not likely to be sensitive enough for detection of mutations in general because of the small size of the gels.

We describe here a nonradioactive high-resolution PCR-SSCP method, F-SSCP, in which fluorescence-labeled primers are used in the PCR. A procedure for fluorescence-labeling of the PCR product and detection of bands of double-stranded DNA in polyacrylamide gel illuminated with UV-light has been described.(6) However, the sensitivity of this method is not high enough for detecting amounts of DNA that are suitable for SSCP analysis. Therefore, we used an automated DNA sequencer for the detection. Use of this automated sequencer also permitted strict control of the gel at any desired temperature, and thus separation could be reproduced at any ambient condition. Moreover, the results can be interpreted quantitatively because the bands are detected as peaks in the fluorogram and their heights are proportional to the intensity of the fluorescence in a wide dynamic range. The direct entry of data into a computer allows objective interpretation of the results; also, data on multiple samples can be processed for further analysis with a reduced possibility of error. These features are especially important for pedigree analysis and linkage studies of polymorphic DNA markers detected by SSCP analysis.

MATERIALS AND METHODS

Primers

The primer sequences for amplification of p53 regions are shown in Table 1. The oligonucleotides were labeled at their 5′ ends with fluorescein-derivatives by two different methods, i.e., by the FluorePrime method (Pharmacia) and with the Oliglow system (ABI). In the FluorePrime method, fluorescein phosphoramidite was used in the last coupling cycle during the synthesis of p53 primers with a DNA Synthesizer 380B (ABI), and the time of coupling was extended to 3 min as suggested previously.(7) The fluorescein-labeled primers, eluted with ammonia, were dried and solubilized in 0.1 M

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TABLE 1 Primer Sequences

Name of primer	Sequence
Fam7U2	Fam-Oliglow-TCATCACACTGGAAGACTCC
Fam8L2	Fam-Oliglow-GTTCCGTCCCAGTAGATTAC
F7U2	F-TCATCACACTGGAAGACTCC
F7U2G	F-GTCATCACACTGGAAGACTCC
F8L2	F-GTTCCGTCCCAGTAGATTAC
F5U1	F-GTTCCTCTTCCTGCAGTACTC
F5L1	F-GCAAATTTCCTTCCACTCGG

Fam-Oliglow is a derivative of fluorescein attached to the 5′ end of an oligonucleotide GAAGAAGC. F is carboxyfluorescein linked to the 5′-nucleotide via a linker and a phosphate. Positions of primers in the p53 gene sequence were described previously.(9) Fragment lengths amplified were: Fam7U2–Fam8L2, 155 bp; F7U2–F8L2, 139 bp; F7U2G–F8L2, 140 bp; F5U1–F5L1, 326 bp.

triethylamine acetate; the full-length oligonucleotides with attached fluorescein were purified by passage through an Oligonucleotide Purification Cartridge (ABI) according to the protocol recommended by the supplier. Some primers synthesized had extra nucleotides at their 5′ ends and were coupled with fluorescein-phosphoramidite as shown in Table 1. For the Oliglow system, "Splint" sequences (14-mer) were synthesized to obtain 8 nucleotides at the 5′ ends that were complementary to "Fam-Oliglow" and 6 nucleotides at the 3′ ends that were complementary to 5′ ends of p53 primers. The p53 primers, Fam-Oliglow, and Splint were ligated at concentrations of 20, 60, and 40 pmoles in 10 μl. The ligation products were used for the PCR without purification.

PCR and SSCP

The target sequences were amplified by the PCR in 5 μl of solution containing 0.1 μM concentrations of the two fluorescent primers, 10 μM concentrations of dATP, dCTP, dGTP, and TTP, 50 ng of human genomic DNA, and 0.125 unit of AmpliTaq (Perkin-Elmer Cetus) in the buffer recommended for the enzyme. The mixtures were subjected to PCR as described previously.(8) The products were diluted with 100 μl of formamide dye solution, heated at 80°C for 5 min, and applied (2 μl/lane) to the SSCP gel(8) fitted to an automated DNA sequencer (ALF, Pharmacia). Intron 7 was analyzed using gel without glycerol, and exon 5 was analyzed using gel containing 5% glycerol. During electrophoresis at 30 W, the temperature of the gel was kept at 25°C with a built-in water jacket connected to an external thermostat-regulated water circulator. Conventional PCR-SSCP analyses were performed in the conditions for the PCR as described above, except that 1 μCi of $[\alpha\text{-}^{32}\text{P}]$dCTP (Amersham) was added. Subsequent procedures were as described previously.(8)

RESULTS

Labeling by the FluorePrime Method

The nucleotide adjacent to fluorescein in the FluorePrime labeling method seems to affect the intensity of fluorescence of the bands in F-SSCP. Examples are shown in Figure 1, in which the polymorphism in intron 7 of p53 was analyzed with the DNA of an individual heterozygous for this locus.(9) In this system, the lower strand of the PCR product has been shown to move faster than the upper strand (data not shown). Using an upper-strand primer, F7U2, which has T at its 5′ end, and a lower-strand primer, F8L2, which has G, the signal of the upper strand was much weaker than that of the lower strand (Fig. 1a). This reduction of the signal was overcome by inserting an extra G at the 5′ end of the upper-strand primer (Fig. 1b). The reason for this effect of the 5′ nucleotide is unknown. Perhaps bonds between some nucleotides and fluorescein are unstable during the heating cycles in the PCR, or the efficiency of fluorescence emission is influenced by the interaction between fluorescein and the neighboring bases. To avoid possible reduction of signal intensity, in subsequent experiments we used primer sequences having G at their 5′ end.

We then examined possible mutations in exon 5 of the p53 gene in the DNA from bladder cancer tissues (Fig. 2) using an upper-strand primer, F5U1, which has the fluorescein moiety attached to an extra G residue (Table 1). As shown in Figure 2, DNA from normal tissue gave two peaks, indicating the positions of the two separated strands of the normal allele. DNA from cancerous tissue gave four peaks, two in the same positions as the peaks of the normal allele but with reduced heights and the other two in different positions from the normal ones. This finding indicates that in

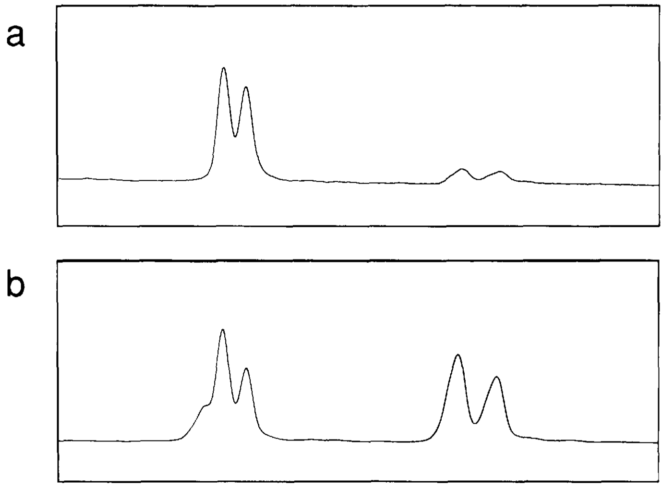

FIGURE 1 Effects of nucleotides adjacent to the fluorophore on fluorescence intensity. Intron 7 of the p53 gene was amplified and analyzed by F-SSCP. DNA from normal tissue of an individual heterozygous with respect to polymorphism in this intron was examined. The upper-strand primers in the PCR were F7U2, which has a T at the 5′ end (*a*), and F7U2G, which has an extra G at the 5′ end (*b*). F8L2, which has an intrinsic G at the 5′ end, was used as a lower-strand primer in both amplification reactions.

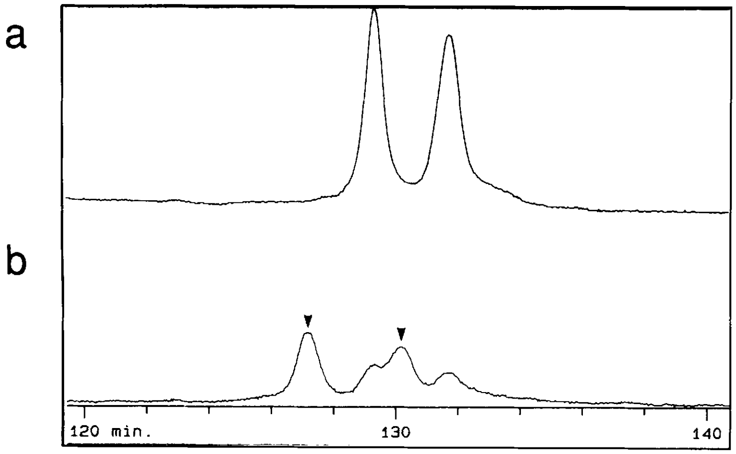

FIGURE 2 Mutation and loss of the normal allele in bladder cancer tissue. Exon 5 of the p53 gene in bladder cancer tissue (*b*) and normal tissue (*a*) was analyzed by F-SSCP with primer F5U1, which has an extra G at the 5′ end, and primer F5L1, which has an intrinsic G at the 5′ end. The two peaks in *a* correspond to complementary strands of normal allele. Arrowheads indicate positions of strands of a mutated allele. Gel containing 5% glycerol was used for electrophoresis.

most cells, the normal allele was lost, and the remaining allele carried the mutation in the examined region.

Labeling by the Fam-Oliglow Method

Figure 3 shows a comparison of F-SSCP using Fam-Oliglow-labeled primers and conventional PCR-SSCP for analysis of polymorphism in intron 7 of the p53 gene⁽⁹⁾ in lung cancer specimens obtained surgically. In the conventional PCR-SSCP, decrease of one allele in the cancerous tissues was seen as a reduction in band intensity of the strands. In F-SSCP, this decrease is demonstrated more convincingly as smaller peaks of these alleles. We found that the relative peak heights of alleles in F-SSCP were reproducible to within 10% in different amplifications (black and green in Fig. 3a and Table 2). Thus, F-SSCP data can be quantitatively interpreted with confidence. Loss of heterozygosity was apparent in both cancerous tissues (blue and red in Fig. 3a), even though a significant amount of the normal allele was seen.

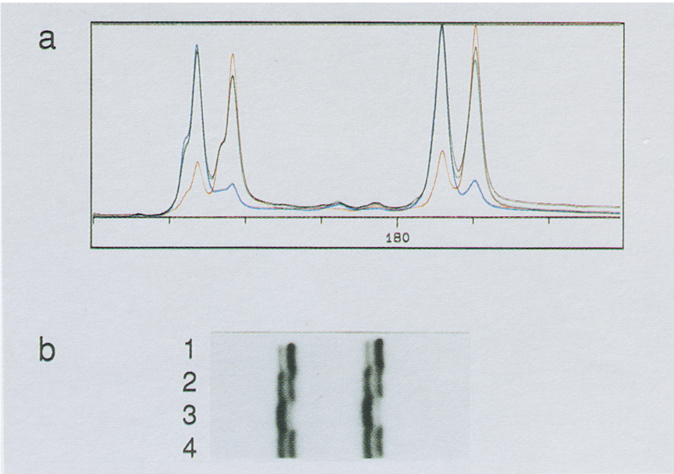

FIGURE 3 Loss of heterozygosity of the *p53* intron 7 in cancerous tissues. Both primers were labeled with Fam-Oliglow and analyzed by F-SSCP (*a*). In *b*, both primers with Fam-Oliglow label were used in the PCR containing [α-^{32}P]dCTP and analyzed by the conventional SSCP as described in the text. The DNA samples examined were from lung cancer tissue of patient 1 (red in *a* and lane 1 in *b*), normal tissue of patient 1 (black in *a* and lane 2 in *b*), lung cancer tissue of patient 2 (blue in *a* and lane 3 in *b*), and normal tissue of patient 2 (green in *a* and lane 4 in *b*).

TABLE 2 Quantitativity of Relative Peak Heights

Sample	Peak 1 (cm)	Peak 2 (cm)	Ratio
1	4.0	4.8	0.83
2	4.0	4.8	0.83
3	3.8	4.7	0.81
4	4.1	4.7	0.87

DNA from normal tissues of 4 individuals was analyzed using Fam7U2–Fam8L2 primer pairs, and the peak heights of the first two peaks were measured. The value of each peak is in centimeters in the chart after subtraction of background.

Apparent partial loss of one allele of the p53 gene in surgical specimens of cancerous tissues is often observed.⁽⁹⁾ The remaining normal allele is likely to be due to contamination of the cancer specimen with the attached normal tissue or with infiltrating lymphocytes.

DISCUSSION

Several commercial kits are available for labeling oligonucleotides with fluorescent dye. Among these, the FluorePrime method is probably the most convenient. In this method, fluorescein phosphoramidite is used at the last cycle of synthesis by the DNA synthesizer. The labeled oligonucleotides are easily freed from unlabeled oligonucleotides by passage through a column containing reversed-phase resin. In the Oliglow method, a common oligonucleotide that carries a fluorophore is ligated to primers with the desired sequence. This method requires an extra oligonucleotide, called Splint, which supports the two oligonucleotides for ligation. The Oliglow method has the advantage that primers already available can be used.

We observed a reduced intensity of fluorescence of fragments having a T at their 5′ end. We do not know if this is true for other sequences. In our case, insertion of an extra G was effective for enhancing the fluorescence intensity. The reason for this difference in the fluorescence intensity is unknown.

Detection of the fluorescent bands with the automated sequencer is quantitative over a wide range of fluorescence intensities. We showed here that loss of heterozygosity of the p53 gene in a cancer specimen obtained surgically can be detected even in the presence of a significant amount of normal alleles, presumably derived from contaminating normal tissues. Use of fluorescent primers in the PCR and detection of the products with the sequencer should also be useful in applications of the PCR other than F-SSCP, e.g., in detecting infections by amplification of a viral or bacterial genome in clinical samples and in detecting cancer cells in the blood by reverse transcription and amplification of mRNAs specifically expressed in cancer cells.

In SSCP analysis, temperature of the gel must be strictly controlled during electrophoresis to obtain reproducible results. For this reason, we chose the Pharmacia ALF automated DNA sequencer, which was the only commercially available fluorescence-based sequencer equipped with water-jacketted electrophoresis apparatus. Using this sequencer, we sometimes observed a small lane-to-lane difference in mobility, although these variations are usually negligible when comparing alleles in nearby lanes. Mobilities can be normalized between lanes by including an internal standard fragment labeled with a different fluorescent dye in each lane. For this, an instrument that is capable of distinguishing two or more colors is required. The currently available Applied Biosystems DNA Sequencer has this capability, but its gel plate is not temperature controllable.

F-SSCP requires an expensive machine. However, its running cost is considerably lower than conventional radioisotopic PCR-SSCP, if many samples are to be analyzed in a long period. Unlike the conventional method, the primers do not need to be periodically labeled once they are synthesized.

Direct entry of the data into a computer is advantageous when a large number of PCR products must be analyzed. In human genome mapping, it is proposed that polymorphic markers be analyzed through the CEPH core family DNA and mapped by linkages among the markers.(10) It is generally believed that many of these polymorphisms will be identified by PCR-based techniques, such as detection of length polymorphisms of CA repeats(11) or other polymorphisms detectable by PCR-SSCP analysis.(12) In these analyses, more than 400 individuals must be genotyped for each polymorphic marker, and the data must be transferred for linkage analysis. This task is currently done largely by eye for interpretation of patterns of X-ray film and by hand for entry of the data through a keyboard for linkage analysis by programs such as LINKAGE.(13) The whole process should become much easier and less error-prone when the data are entered directly into a computer, interpreted there, and processed for calculation. Future development of software for the automated sequencer for use in F-SSCP analysis should include allele identification, an interface for transfer of data to other applications, and, possibly, normalization of the mobility in each lane.

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REFERENCES

1. Hayashi, K. 1991. PCR-SSCP: A simple and sensitive method for detection of mutations in genomic DNA. *PCR Methods Applic.* **1:** 34–38.
2. Orita, M., Y. Suzuki, T. Sekiya, and K. Hayashi. 1989. A rapid and sensitive detection of point mutations and genetic polymorphisms using polymerase chain reaction. *Genomics* **5:** 874–879.
3. Ainsworth, P.J., L.C. Surh, and M.B. Coulter-Mackie. 1991. Diagnostic single strand conformational polymorphism, (SSCP): A simplified non-radioisotopic method as applied to a Tay-Sachs B1 variant. *Nucleic Acids Res.* **19:** 405–406.
4. Dockhorn-Dworniczak, B., B. Dworniczak, L. Brommelkamp, J. Bulles, J. Horst, and W. Bocker. 1991. Non-isotopic detection of single strand conformation polymorphism (PCR-SSCP). *Nucleic Acids Res.* **19:** 2500.
5. Mohabeer, A.J., A.L. Hiti, and W.J. Martin. 1991. Nonradioactive single strand conformation polymorphism (SSCP) using the Pharmacia "PhastSystem". *Nucleic Acids Res.* **19:** 3154.
6. Chehab, F.F. and Y.W. Kan. 1989. Detection of specific DNA sequences by fluorescence amplification: A color complementation assay. *Proc. Natl. Acad. Sci.* **86:** 9178–9182.
7. Hawkins, T.L. and J.E. Sulston. 1991. Automated fluorescent primer walking. *Technique* **2:** 307–310.
8. Mashiyama, S., T. Sekiya, and K. Hayashi. 1990. Screening of multiple samples for detection of sequence changes. *Technique* **2:** 304–306.
9. Mashiyama, S., Y. Murakami, T. Yoshimoto, T. Sekiya, and K. Hayashi. 1991. Detection of p53 gene mutations in human brain tumors by single-strand conformation polymorphism analysis of polymerase chain reaction products. *Oncogene* **6:** 1313–1318.
10. Dauset, J., H. Cann, D. Cohen, M. Lathrop, J.-M. LaLouel, and R. White. 1990. Centre d'Etude du Polymorphisme Humain (CEPH): Collaborative Genetic Mapping of the Human Genome. *Genomics* **6:** 575–577.
11. Litt, M. and J.A. Luty. 1989. A hypervariable microsatellite revealed by *in vitro* amplification of a dinucleotide repeat within the cardiac muscle actin gene. *Am. J. Hum. Genet.* **44:** 397–401.
12. Iizuka, M., S. Mashiyama, M. Oshimura, T. Sekiya, and K. Hayashi. 1992. A generic method of obtaining polymorphic sequence tagged sites: Polymerase chain reaction-single strand conformation polymorphism analysis of anonymous *Alu* repeats on chromosome 11. *Genomics* **12:** 139–146.
13. Lathrop, M., J.-M. LaLouel, C. Julier, and J. Ott. 1985. Multilocus linkage analysis in humans: Detection of linkage and estimation of recombination. *Am. J. Hum. Genet.* **37:** 482–498.

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