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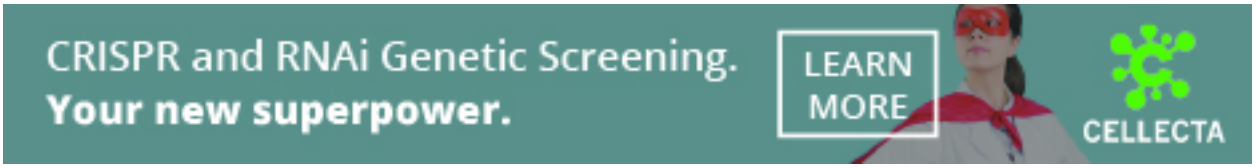

Multiple Fluorescence-based PCR–SSCP Analysis with Postlabeling

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Multiple fluorescence-based PCR single-strand conformation polymorphism (MF–PCR–SSCP) with postlabeling was developed. The target sequence was amplified by PCR using unlabeled primers. Free dNTPs were removed from the amplified products by ethanol precipitation. The dNTPs at the 3′ ends of the amplified DNA fragments were exchanged with fluorescent dUTPs or ddNTPs using Klenow fragment of DNA polymerase I. The DNA fragments labeled with fluorescent dUTPs or ddNTPs were heat denatured and applied to a nondenaturing polyacrylamide gel set on an automated DNA sequencer with a gel temperature-controlling system. The image data were analyzed by the computer program Genescan 672. By use of MF–PCR–SSCP with postlabeling, seven different single base mutations of the human K-*ras* oncogene were detected even under one electrophoresis condition.

Single-strand conformation polymorphism (SSCP) analysis was developed to detect mutations and polymorphisms in genomic DNA.[1] The combination of SSCP with PCR gave birth to PCR–SSCP[2] and its applicability was expanded dramatically. Using PCR–SSCP, DNA alterations, including even one point mutation in cancer and hereditary diseases, were detected successfully.[2–5] Furthermore, SSCP brought about several applications, such as PCR–SSCP with endonuclease digestion,[6,7] RNA–SSCP (rSSCP),[8] dideoxy fingerprinting (ddF),[9] SSCP with silver staining,[10] amplification refractory mutation system (ARMS)–SSCP,[11] fluorescence-based PCR–SSCP (F–SSCP),[12,13] and multiple fluorescence-based PCR–SSCP (MF–PCR–SSCP).[14,15]

PCR–SSCP with endonuclease digestion was developed to overcome the limitations of SSCP in relation to the length of DNA.[6,7] SSCP using RNA (rSSCP) was shown to give better results than SSCP using DNA.[8] Because SSCP was influenced by the length of DNA fragments and the position of mutations, ddF was developed by combining SSCP with DNA sequencing.[9] Variable sizes of DNA fragments and positions of mutations were analyzed at the same time, and the sensitivity of mutation detection was increased.

The necessary use of radioisotopes is a disadvantage for routine examination in the use of SSCP, despite its sensitivity. Silver staining was introduced to replace radioisotopes for band detection.[10] The fluorescent ARMS–SSCP was performed using two different fluorescence-labeled primers, and bands were visualized by UV transillumination.[11] Hayashi et al.[12] and Takahashi-Fujii et al.[13] developed F–SSCP using an automated DNA sequencer and a fluorescence-based image analyzer, respectively. In their systems, however, band detection was dependent on a single fluorescence derived from primers labeled with a single fluorescent dye. Ellison et al.[14] reported a method using multiple fluorescence-labeled primers and detecting with an Applied Biosystems model 373A DNA sequencer (Perkin Elmer, Applied Biosystems Division, Foster City, CA) without controlling gel temperature. We designed and attached a gel temperature-controlling system to the same model DNA sequencer and developed a sensitive MF–PCR–SSCP.[15]

Currently, two types of fluorescent DNA sequencing are used widely: dye primer and dye terminator sequencing. In dye terminator sequencing, fluorescence-labeled dideoxynucleotides (ddNTPs) are incorporated into DNA fragments with DNA polymerases. Various fluorescence-labeled ddNTPs were synthesized for dye terminator sequencing.[16] We thought it possible to label PCR–amplified DNA fragments at the 3′ end with fluorescence-labeled nucleotides using DNA polymerases which commit the replacement reaction at the 3′ end of DNA fragments.

Here, we describe a nonradioactive, high-resolution MF–PCR–SSCP with postlabeling. To establish this method we developed a new apparatus, a modification of model 373A automated DNA sequencer, to control gel temperature. Also, we developed a method of postlabeling PCR-amplified DNA fragments with fluorescent dyes after PCR.

MATERIALS AND METHODS

Automated DNA Sequencer and Control of Gel Temperature

We developed a device to control gel

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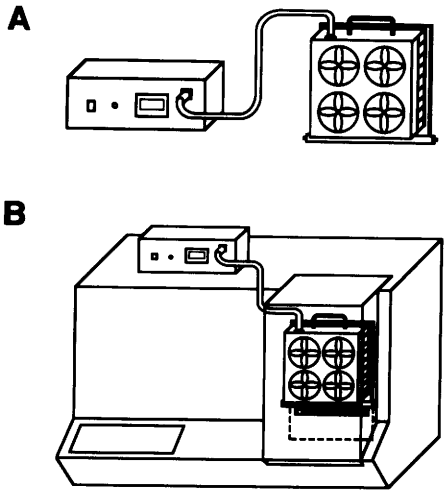

FIGURE 1 The gel temperature-controlling system. This system consists of a control unit and a one-touch attachment unit. (*A*) A regulator unit (*left*) and a one-touch attachment unit (*right*). (*B*) The gel temperature-controlling system can be attached to the Applied Biosystems 373A DNA sequencer by hooking the one-touch attachment unit on the front surface of the gel. It can be removed as easily as it is attached.

temperature for the previous model of Applied Biosystems 373A DNA sequencer.[15] The modified model of the sequencer can be used for three different gel sizes—6, 12, and 24 cm. Because this sequencer and its original model had no means for con-trolling gel temperature, we designed andattached such a system to the sequencer in cooperation with Astec (Fukuoka, Japan) and Perkin-Elmer Japan, Applied Biosystems Division (Osaka and Fukuoka, Japan). As shown in Figure 1, the system consists of a regulator unit and a one-touch attachment unit. The surface of the attachment unit is temperature controlled with Peltier elements and placed onto the surface of the front glass plate of the gel. Thus, the gel temperature is controlled strictly between 10°C and 60°C±0.5°C.

Cell Lines and DNA Isolation

Seven human tumor cell lines, A549, Lu65, MDA-MB231, PANC1, PSN1, SW480, and SW1116, each carrying a different mutation of the K-*ras* gene, were the source of DNA samples as described previously.[15] Peripheral white blood cells were obtained from a healthy donor. High molecular weight DNAs were prepared from the cultured tumor cell lines and the peripheral white blood cells.

TABLE 1 Primers Used for MF–PCR–SSCP with Postlabeling

Primer name	Sequence[a]	Note[b]
oRB492	5′–ACCTTATGTGTGACATGTTC–3′	S
oRB493	5′–TGAAAATGGTCAGAGAAACC–3′	AS
oRB1050	5′–*T*GAGGCCTGCTGAAAATGACTG–3′	S, R6G–ddATP
oRB1120	5′–*A*GAGGCCTGCTGAAAATGACTG–3′	S, TAMRA–ddTTP, [F]dUTPs
oRB1051	5′–*C*GAAAGAATGGTCCTGCACCAG–3′	AS, R110–ddGTP
oRB1078	5′–*A*GAAAGAATGGTCCTGCACCAG–3′	AS, TAMRA–ddTTP, [F]dUTPs
oRB1121	5′–*G*TAAAGAATGGTCCTGCACCAG–3′	AS, 6-ROX–ddCTP
oRB1122	5′–*T*GAAAGAATGGTCCTGCACCAG–3′	AS, R6G–ddATP

[a]The 5′-end bases in italics indicate those introduced for postlabeling. The underlined 5′ penultimate bases indicate those introduced to stop the exchange reaction by non-fluorescence-labeled nucleotides.
[b](S) Sense strand primer; (AS) antisense strand primer. Fluorescent nucleotides are expected to label the 3′ end of the complementary sequence of the primer. ([F]dUTP) Nucleotides including R6G–dUTP, R110–dUTP, and TAMRA–dUTP.

PCR Amplification

Primers used in this experiment are listed in Table 1. These primers were synthesized using the Applied Biosystems 392 DNA/RNA synthesizer. To obtain the pure PCR product and to repeat the experiment with a small amount of DNA sample, the 175-bp DNA fragment containing K-*ras* codons 12 and 13 was amplified by the nested PCR. The first PCR was performed using a pair of primers, oRB492–oRB493, and 100 ng of DNA was

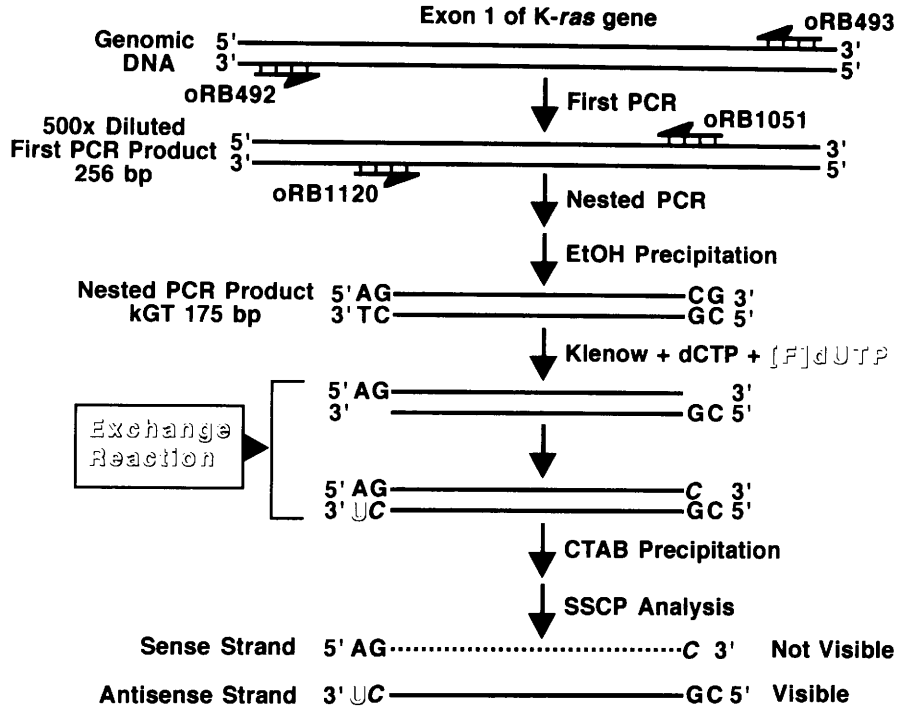

FIGURE 2 Flowchart of the MF–PCR–SSCP with postlabeling. The 175-bp DNA fragment of kGT containing K-*ras* codons 12 and 13 was amplified by nested PCR using sets of primers oRB492-oRB493 and oRB1120-oRB1051. The free dNTPs were removed by ethanol precipitation. The antisense strand of kGT was labeled with 0.5 μM R6G–dUTP using the Klenow fragment of DNA polymerase I. To stop 3′ → 5′ exonuclease activity of the Klenow fragment at the 3′-penultimate base, unlabeled dCTP was added to the reaction mixture to a final concentration of 125 μM. Unincorporated [F]dUTP was removed by CTAB precipitation. The postlabeled PCR product was subjected to SSCP analysis. In this case, only the 3′-end base of the antisense strand was exchanged with [F]dUTP. The antisense strand was thus visible, but the sense strand was not.

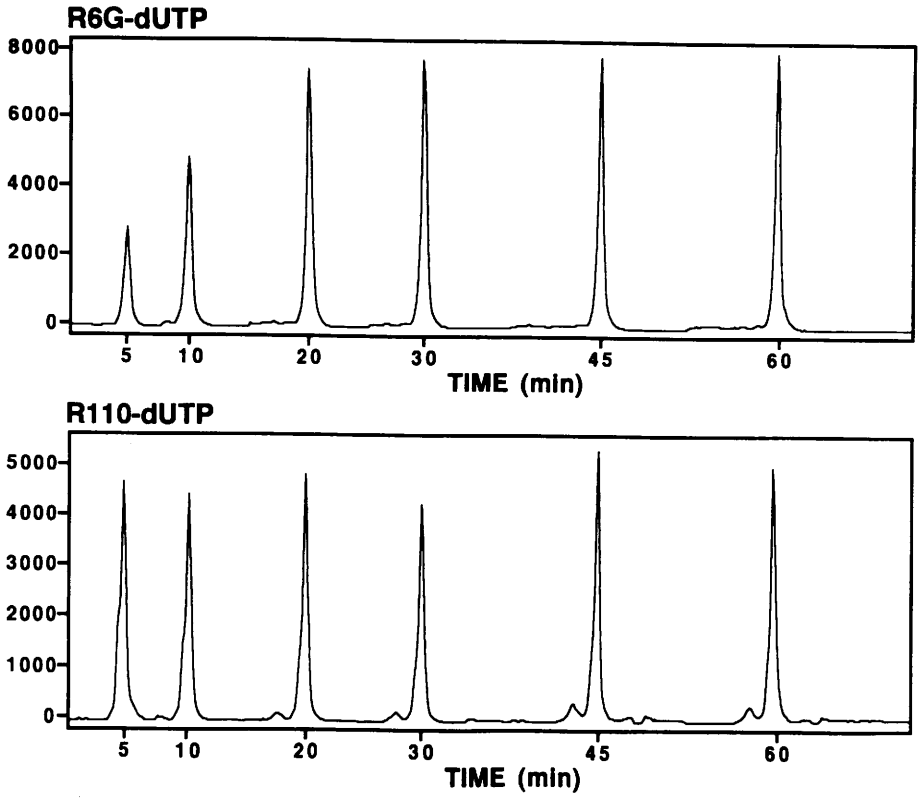

FIGURE 3 Time course of fluorescence labeling. The antisense and sense strands of 175-bp DNA fragments of the K-*ras* gene were labeled using 2 units of Klenow fragment with 0.5 μM R6G–dUTP and 0.5 μM R110–dUTP, respectively. The labeling reaction was stopped at the indicated time. Electrophoresis was performed on a 6% polyacrylamide/7 M urea gel.

extracted from either a cell line or peripheral white blood cells as a template, in a total volume of 10μl. The second PCR was carried out using an indicated pair of primers and the product of the first PCR (500×) as a template in a total volume of 50 μl. The reaction mixture of the first and second PCRs contained 1 μM each of primers; 200 μM of dATP, dCTP, dGTP, and dTTP; the indicated DNA as a template; and 0.025 U/μl of *Taq* DNA polymerase (Perkin-Elmer, Norwalk, CT) in buffer with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% (wt/vol) gelatin. Thirty reaction cycles at 94°C, 55°C, and 72°C for 1, 1, and 2 min, respectively, were propagated in a Program Temp Control System PC-700 (Astec). The first denaturation at 94°C was extended to 3 min, and the last polymerization at 72°C was extended to 10 min to complete the elongation reaction. After the second PCR amplification, 10 μl of the PCR products was electrophoresed on a 1% agarose gel, and the amplification of 175-bp DNA fragments was confirmed. The remaining 40 μl of the PCR products was ethanol precipitated using ammonium acetate as a salt and dissolved in 30 μl of deionized, autoclaved water (DA water). The amount of amplified DNA fragments was determined using a DU-64 spectrophotometer (Beckman, Fullerton, CA).

Postlabeling with Fluorescent Dyes

The fluorescent dUTPs ([F]dUTPs) of R6G–dUTP (green, 100 μM), R110–dUTP (blue, 100 μM), and TAMRA–dUTP (yellow in gel image and black in electrophoretogram, 400 μM) were kindly provided by Perkin-Elmer. The fluorescent ddNTPs ([F]ddNTPs) of R6G–ddATP (green, 15 μM), 6-ROX–ddCTP (red, 450 μM), R110–ddGTP (blue, 4 μM), and TAMRA–ddTTP (yellow in gel image and black in electrophoretogram, 900 μM) were from the *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (Perkin-Elmer catalog no. 401113).

A flowchart of MF–PCR–SSCP with postlabeling is shown in Figure 2. The amplified DNA fragment of 0.1 μg was mixed with the desired [F]dUTPs or [F]ddNTPs and 125 μM indicated unlabeled dNTPs in the buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 100 mM NaCl in a total volume of 20 μl. The indicated units of Klenow fragment of DNA polymerase I (Takara, Kyoto, Japan) were added and incubated at 37°C for an appropriate period. The exchange reaction proceeded as follows. The 3′ end nucleotide was removed by the 3′ → 5′ exonuclease activity of DNA polymerase I Klenow fragment and filled with the fluorescence-labeled nucleotide by the 5′ → 3′ DNA polymerase activity of Klenow fragment. The excess amount of unlabeled dNTP corresponding to the 3′-penultimate base was added to stop the exchange reaction at this position. To remove the free [F]dUTPs or [F]ddNTPs, 2.5 μl of 5% cetyltrimethylammonium bromide (CTAB) was added and centrifuged at 16,000*g* at room temperature for 3 min. The supernatant was discarded. Fifty microliters of 1.2 M NaCl and 125 μl of 99.5% ethanol were added and centrifuged again at 16,000*g* at room temperature for 3 min. The pellet was washed with 500 μl of 70% ethanol and centrifuged under the same conditions. The precipitate was dissolved with 5–20 μl of a mixture of formamide and EDTA (formamide/50 mM EDTA, 5:1).

SSCP Analysis

The fluorescence-labeled DNA fragment in the mixture of formamide and EDTA was heated at 90°C for 3 min, and cooled immediately on ice. From 1 to 5 μl of this solution was applied to a nondenaturing polyacrylamide gel set to a DNA sequencer equipped with the temperature-controlling system. Genescan-2500 ROX (Perkin-Elmer) was used as an internal DNA size marker. To make 10–14% polyacrylamide gel, 50 ml of the SSCP gel mixture containing 12.5–17.5 ml of 40% acrylamide (acrylamide/*N,N*-methylenebisacrylamide; 50:1), 5 ml of 10× TBE buffer, 1050 μl of 3% ammonium persulfate, and 30 μl of *N,N,N′,N′*-tetramethylethylenediamine (TEMED) was used, with 5 ml of glycerol when indicated. The gel temperature was controlled strictly within the range from 15°C to 30°C. Electrophoresis was performed in 1× TBE buffer at 30 W, and the data were collected and analyzed us-

ing Genescan 672 software (Perkin-Elmer).

RESULTS

Time Course of Fluorescence Labeling

The 175-bp DNA fragments of kGT (k = K-*ras* gene; G and T = the sense and antisense strands labeled with [F]ddGTP and [F]dUTPs, respectively) of the K-*ras* gene were amplified with a set of primers, RB1120–oRB1051 (Fig. 2). The antisense strand of kGT was labeled with 0.5 μM R6G–dUTP. To stop 3′ → 5′ exonuclease activity of DNA polymerase I Klenow fragment at the 3′-penultimate base, unlabeled dCTP was added to the reaction mixture in a final concentration of 125 μM. The labeling reactions were performed using 2 units of Klenow fragment at 37°C for 5, 10, 20, 30, 45, and 60 min. Electrophoresis was carried out on a 6% polyacrylamide/7 M urea gel. As shown in Figure 3 (top), kGT was labeled with R6G–dUTP at 5 min. The labeling reaction reached the stable equilibrium at 20 min, and the intensity of the label remained at the same level thereafter.

The 175-bp DNA fragment of kTA of the K-*ras* gene was amplified with primers, oRB1050–oRB1078. The sense strand of kTA was labeled with 0.5 μM R110–dUTP. The kTA labeling reaction with R110–dUTP reached equilibrium as early as 5 min, but the fluorescence intensity was about one-half that of kGT labeled with R6G–dUTP (Fig. 3, bottom).

Effect of Klenow Fragment Dose on Labeling

The dose of DNA polymerase I Klenow fragment was varied as 0.5, 1.0, 2.0, and 4.0 units in the postlabeling reactions of kGT and kTA with 0.5 μM R6G–dUTP and 0.5 μM R110–dUTP, respectively. Unlabeled dCTP was used to stop 3′ → 5′ exonuclease activity of Klenow fragment at the 3′-penultimate base. The reaction mixture was incubated at 37°C for 30 min. Electrophoresis was performed on a 6% polyacrylamide/7 M urea gel. The fluorescence was dose dependent and peaked at 2.0 units, no further enhancement was observed up to 4.0 units (Fig. 4).

Effect of Various [F]dUTPs or [F]ddNTPs on Labeling

In addition to DNA fragments of kGT and kTA, the 175-bp DNA fragments of kAT, kCA, and kGA of the K-*ras* gene were amplified with primers oRB1120–oRB1122, oRB1050–oRB1121, and oRB1050–oRB1051, respectively. Unlabeled dCTP for kAT, kGA, kGT, and kTA and unlabeled dATP for kCA were used to stop 3′ → 5′ exonuclease activity of Klenow fragment at the 3′-penultimate base. DNA fragments (0.1 μg) were used, and sense strands were labeled. Each fluorescent nucleotide was used at the indicated concentration to label a DNA fragment. The reaction mixture contained 2 units of Klenow fragment, and the incubation was carried out at 37°C for 30 min.

The results are shown in Figure 5. R6G–dUTP showed sharp, high peaks in the examined range of 0.05–10 μM. R110–dUTP showed a peak with the half maximum height at 0.05 μM and reached a plateau at 0.25 μM. From 2.5 μM, R110–dUTP exhibited multiple peaks. In TAMRA–dUTP, the peak showed a dose-dependent increase up to 20 μM, but even the highest peak was about one-fourth the height of that in R6G–dUTP. Because the original concentrations of R6G–ddATP and R110–ddGTP were low, we examined them in the ranges of 0.075–1.5 and 0.02–0.4 μM, respectively. In those ranges, DNA fragments of kAT and kGA were labeled in a dose-dependent fashion by R6G–ddATP and R110–ddGTP, respectively. Although we checked the efficacy of 6-ROX–ddCTP and TAMRA–ddTTP in postlabeling DNA fragments of kCA and kTA in the range of 2.25–45 and 4.5–90 μM, respectively, we could not find any evidence of DNA labeling.

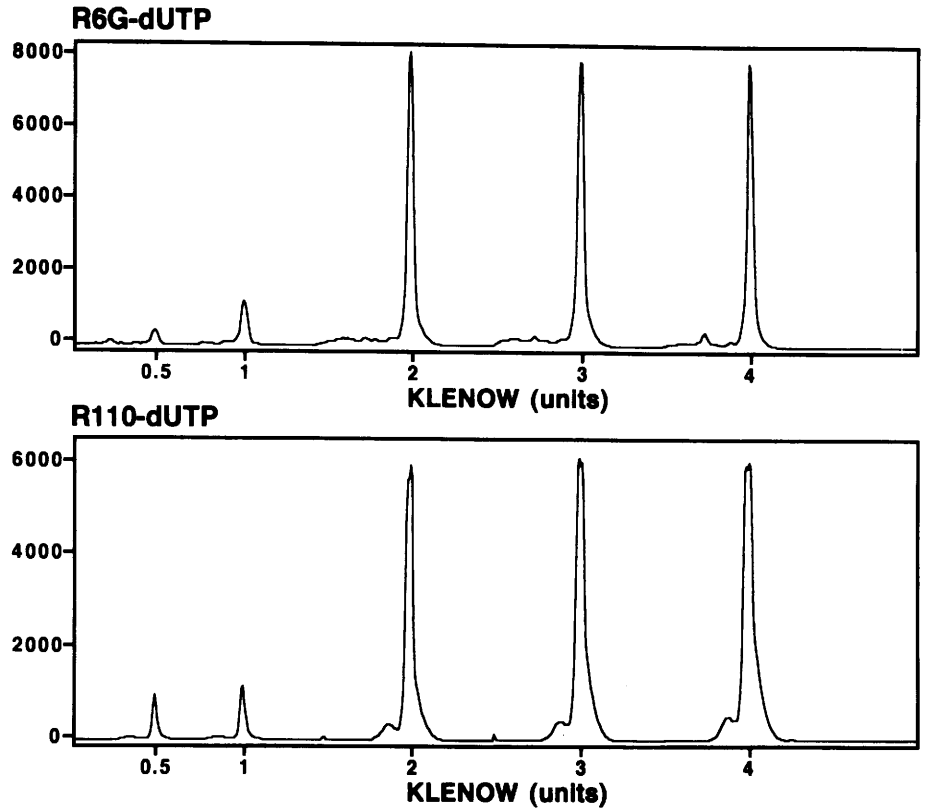

FIGURE 4 Effect of Klenow fragment dose on postlabeling. The dose of Klenow fragment was varied as 0.5, 1.0, 2.0, 3.0, and 4.0 units in the postlabeling reaction of the antisense and sense strands of 175-bp DNA fragments of the K-*ras* gene with 0.5 μM R6G–dUTP and 0.5 μM R110–dUTP, respectively. The incubation was at 37°C for 30 min, and electrophoresis was carried out on a 6% polyacrylamide/7 M urea gel.

Effect of Different Fluorescent Labeling on SSCP Analysis

The 175-bp DNA fragments of the K-*ras* gene were amplified using primers oRB1050–oRB1078 from genomic DNAs, which were isolated from peripheral white blood cells from a normal individ-

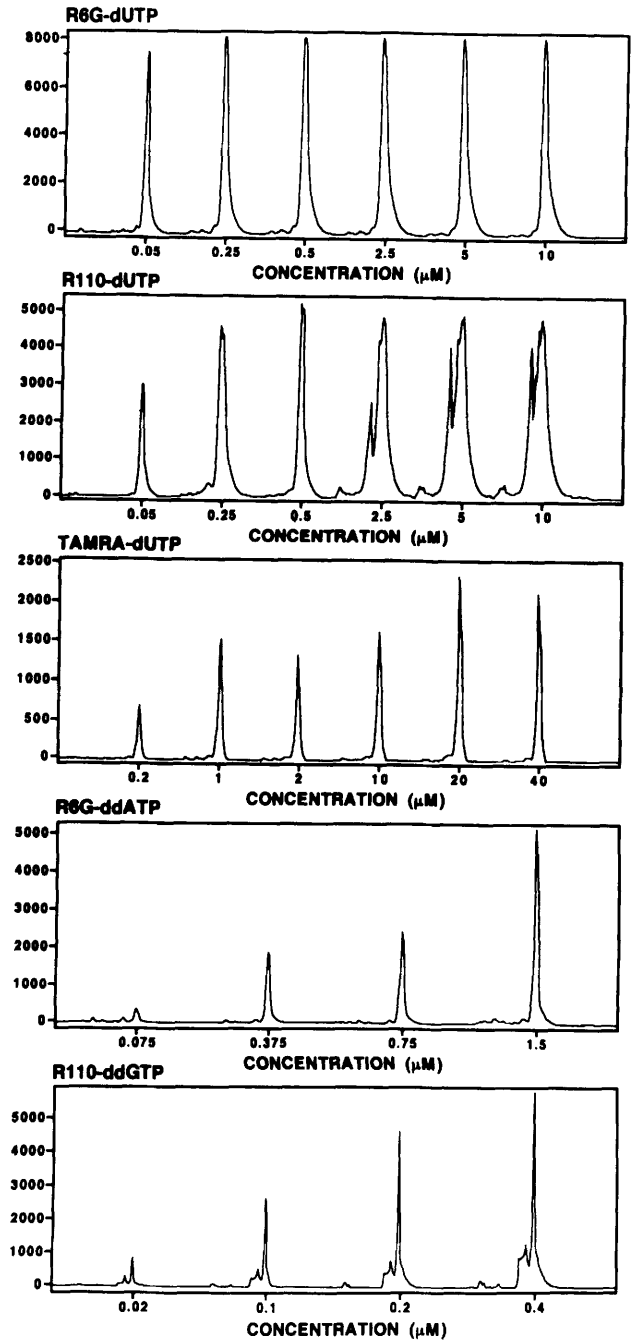

FIGURE 5 Efficacy of various [F]dUTPs and [F]ddNTPs on postlabeling. The 175-bp DNA fragments of the K-*ras* gene were amplified with indicated primers. The sense strand of each DNA fragment was postlabeled with the indicated fluorescent nucleotides using 2 units of Klenow fragment. The incubation was at 37°C for 30 min, and electrophoresis was carried out on a 6% polyacrylamide/7 M urea gel.

ual and seven tumor cell lines. The sense strands of these DNA fragments were postlabeled with 0.5 μM R6G–dUTP, 0.5 μM R110–dUTP, or 2.0 μM TAMRA–dUTP. DNA fragments labeled with three different fluorescent dyes were mixed and electrophoresed on a 12% polyacrylamide gel. The gel length was 6 cm, and the gel temperature was kept at 20°C. As shown in Figure 6, SSCP profiles were almost the same, when three different [F]dUTPs were used for postlabeling. Thus, the different fluorescent labeling showed little effect on the migration of single-stranded DNA in SSCP analysis.

SSCP Analysis with Coelectrophoresis

The 175-bp DNA fragments of the K-*ras* gene were amplified using primers oRB1050–oRB1078. The sense strand of the normal control was labeled with 0.5 μM R110–dUTP, and the sense strands of the control and seven tumor cell lines were labeled with 0.5 μM R6G–dUTP. The R110–dUTP-labeled normal control and the R6G–dUTP-labeled DNA fragments of normal control and tumor cells were mixed, and SSCP analysis was performed. Genescan-2500 ROX was used as an internal DNA size marker. As shown in Figure 7, all seven mutations of the K-*ras* gene were clearly detected as different migration patterns on a 12% polyacrylamide gel in the 6-cm gel at 20°C.

Simultaneous Postlabeling of Sense and Antisense Strands

The DNA fragments of the K-*ras* gene amplified with a set of primers, oRB1050–oRB1078, were labeled simultaneously with 0.05 μM R110–dUTP and 0.075 μM R6G–ddATP. Unlabeled dCTP was used to stop 3′ → 5′ exonuclease activity of Klenow fragment at the 3′-penultimate base. Sense strands labeled with R110–dUTP and antisense strands labeled with R6G–ddATP were shown as blue and green bands, respectively, on the gel image (Fig. 8). As shown in Figure 8, all tumor cell lines show different migration patterns compared with the control. Thus, both sense and antisense strands were labeled with different fluorescent dyes at the same time and successfully applied for SSCP analysis. However, the control showed three blue bands and one green band (Fig. 8, lane 1). Other samples also showed two to three blue bands and one green band for each allele. We electrophoresed these samples on 6% polyacrylamide/7 M urea gel and detected two major blue bands and one green band for each sample (data not shown). This result suggests that several Ts at the 3′ end of the sense strand were replaced with R110–dUTP and an A at the 3′ end of the antisense strand was replaced with R6G–ddATP.

DISCUSSION

We have developed and successfully used PCR–SSCP analysis in the detection of DNA alterations in cancer and hered-

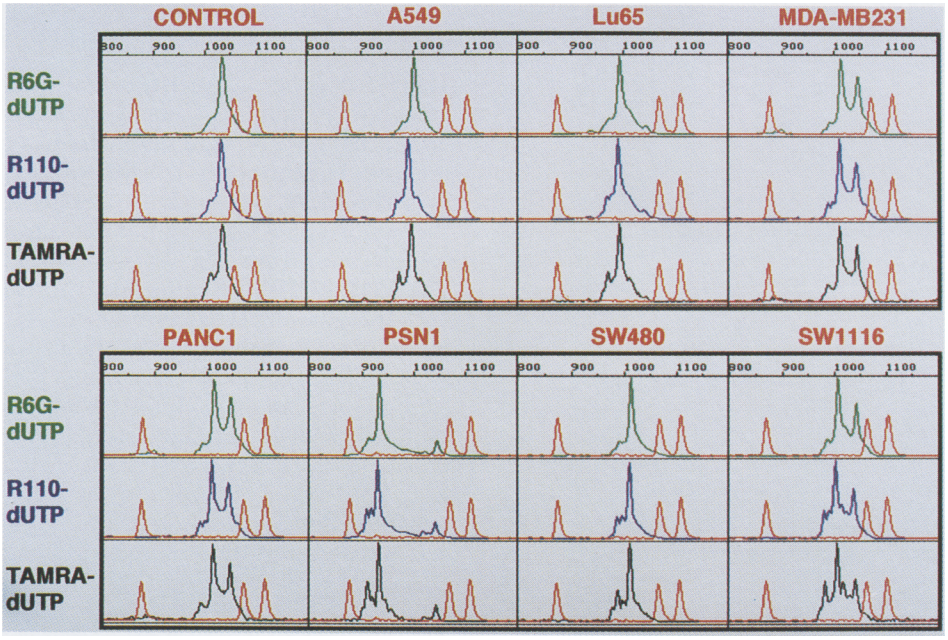

FIGURE 6 Effect of different fluorescent dyes on SSCP analysis. The 175-bp DNA fragments of the K-*ras* gene were amplified from genomic DNA isolated from peripheral white blood cells from a normal individual and seven tumor cell lines using primers oRB1050–oRB1078. The sense strands of these DNA fragments were postlabeled with 0.5 μM R6G–dUTP, 0.5 μM R110–dUTP, or 2 μM TAMRA–dUTP using 2 units of Klenow fragment at 37°C for 30 min. DNA fragments labeled with three different fluorescent dyes were mixed, and SSCP analysis was performed using a 12% polyacrylamide gel. The gel size was 6 cm, and the gel temperature was kept at 20°C. The red peaks indicate the internal standard DNA markers, Genescan-2500 ROX.

itary diseases.[1,3–7] To overcome the disadvantage of PCR–SSCP based on radioisotope labeling, we developed multiple fluorescence-based MF–PCR–SSCP analysis.[15] The Applied Biosystems 373A DNA sequencer, for which we originally developed MF–PCR–SSCP analysis, was improved to permit the use of three different sizes of gels: 6, 12, and 24 cm. Because our original gel temperature-controlling system with a water-circulating setup did not fit the new model, we upgraded the original system (Fig. 1) and further developed the MF–PCR–SSCP technique with postlabeling.

The one-touch attachment unit of the new gel temperature-controlling system weighs 2.5 kg. It attaches to and releases from the front of the gel plate easily. This system makes it possible to control the gel temperature strictly between 10°C and 60°C±0.5°C.

Synthesis of fluorescence-labeled primer is time-consuming and expensive, and fluorescence-labeled primers do not work well in some cases. We tried to develop a method to label DNA fragments with fluorescent dyes after PCR amplification. First, we attempted to postlabel DNA fragments with T4 DNA polymerase without success. Next, we tried to postlabel DNA fragments with the Klenow fragment of DNA polymerase I. The Klenow fragment effectively incorporated all three [F]dUTPs (R6G–dUTP, R110–dUTP, and TAMRA–dUTP), and two [F]ddNTPs (R6G–ddATP and R110–ddGTP) and successfully postlabeled DNA fragments (Fig. 5). We amplified DNA fragments of the K-*ras* gene by the nested PCR technique. However, if the PCR product was pure and enough DNA sample was available, the amplification by nested PCR was not necessary.

The differences in fluorescent dyes had little influence on the migration in SSCP (Fig. 6). The mixture of R110–dUTP-labeled control (blue) and R6G–dUTP-labeled tumor samples (green) electrophoresed in the same lane enabled the easy detection of the mutated K-*ras* genes, as the green peaks migrated differently from the blue peak (Fig. 7). Ellison et al.[14] amplified control DNA with TAMRA-labeled primer and mutated DNAs with FAM- and JOE-labeled primers. They showed that SSCP with co-electrophoresis was more effective than SSCP alone.

We found that the larger the gel size, the better the resolution of SSCP analysis became (data not shown). However, it took more time to get results with the larger gel. In our previous study,[15] we showed that 20°C was the optimum temperature in the range from 10°C to 40°C for SSCP analysis of the K-*ras* gene. In this study we controlled the gel temperature at 15°C, 20°C, 25°C, and 30°C. We found that 20°C was the optimum temperature (data not shown). The effects of polyacrylamide concentration and glycerol in the gel on the SSCP analysis were studied (data not shown). As polyacrylamide concentration was increased to 14%, the resolution of mobility shifts improved. On the other hand, a 14% polyacrylamide gel caused a significant distortion of the SSCP profile. Adding 5% glycerol to the gel did not improve the resolution of SSCP analysis. Based on these results, the screening of K-*ras* mutations can be performed using 12% polyacrylamide gel in the 6-cm gel at 20°C. The larger gel, of 12 or 24 cm, can be used to confirm the K-*ras* gene mutations.

We labeled sense and antisense strands successfully with different fluorescent dyes (Fig. 8). The sense strand labeled with R110–dUTP showed multiple bands. This was probably the result of the sequence of the 5′ end of primer oRB1078, with 5′-AGAAAGAAT-3′. The exchange reaction with Klenow fragment could not be stopped at the penultimate base, even with the excess amount of unlabeled dCTP. Thus, to make a sense strand appear as a single band, a few bases (except A) are needed beyond the penultimate base, and unlabeled dNTPs (except for dTTP) must be added to the reaction mixture.

MF–PCR–SSCP with postlabeling is attractive for several reasons: Fluorescence-labeled primers are not required; internal standards that are DNA markers and a control DNA labeled with different fluorescent dye from samples are available; the image data are entered directly into the computer; and DNA is labeled with nonisotopic dyes. Because of these advantages, MF–PCR–SSCP with postlabeling can be used routinely to analyze a large number of DNA samples as a part of genetic diagnosis.

ACKNOWLEDGMENTS

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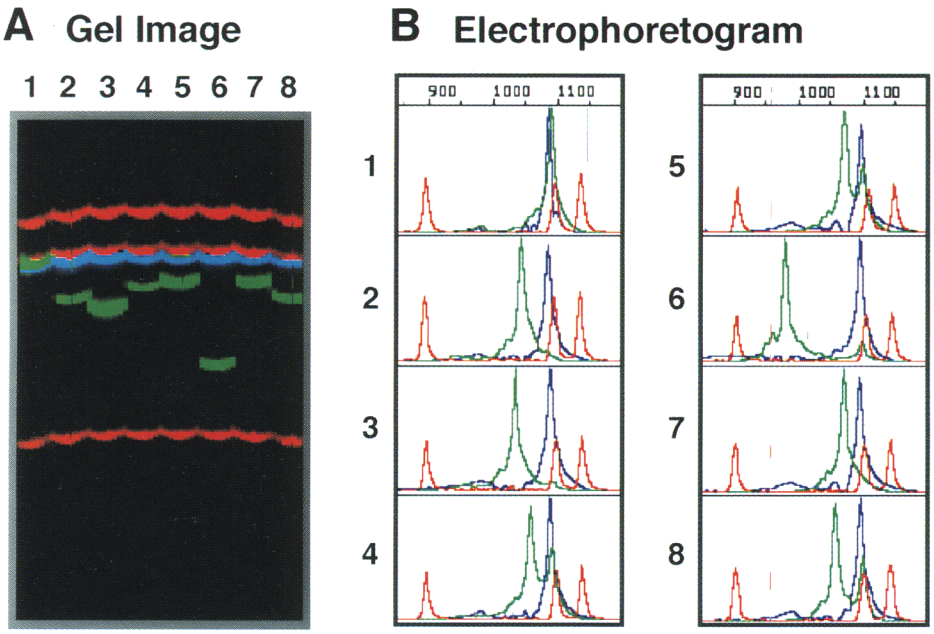

FIGURE 7 SSCP analysis with coelectrophoresis. The 175-bp DNA fragments of the K-*ras* gene were amplified using primers oRB1050–oRB1078. The sense strand of the normal control was labeled with 0.5 μM R110–dUTP, and the sense strands of the normal control and seven tumor cell lines were labeled with 0.5 μM R6G–dUTP. The R110–dUTP-labeled normal control and the R6G–dUTP-labeled DNA fragments of normal control and tumor cells were mixed and SSCP analysis was performed. Genescan-2500 ROX was used as an internal DNA size marker. The acrylamide concentration was 12%, and the length of the gel was 6 cm. The gel temperature was controlled at 20°C. (*A*) Gel image; (*B*) electrophoretogram. (*A*) The blue, green, and red bands indicate the normal control, samples, and internal standard DNA size markers of Genescan-2500 ROX, respectively. (*B*) The blue, green, and red peaks indicate the normal control, samples, and Genescan-2500 ROX, respectively. (Lanes *1–8* in *A* and *B*) Normal control, A549, Lu65, MDA-MB231, PANC1, PSN1, SW480, and SW1116, respectively.

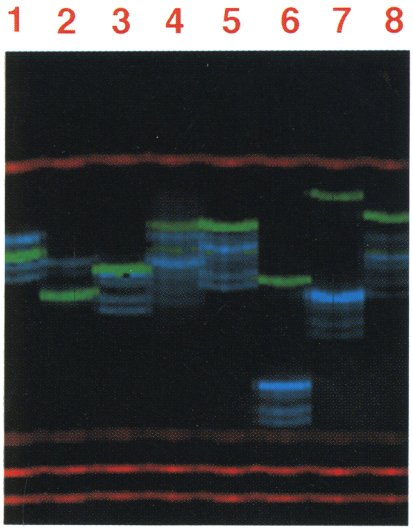

FIGURE 8 Simultaneous postlabeling of sense and antisense strands. A SSCP gel image was shown for the K-*ras* gene of a normal control and seven tumor cell lines, of which sense and antisense strands were postlabeled with 0.05 μM R110–dUTP and 0.075 μM R6G–ddATP, respectively. The 175-bp DNA fragments of the K-*ras* gene were amplified using primers oRB1050–oRB1078. Electrophoresis was performed using a 10% polyacrylamide gel at 20°C. The size of the gel was 12 cm. The blue and green bands indicate sense and antisense strands, respectively. The red bands indicate Genescan-2500 ROX. (Lanes *1-8*) SSCP of a normal control, A549, Lu65, MDA-MB231, PANC1, PSN1, SW480, and SW1116, respectively.

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