GENOME METHODS

One-tube Post-PCR Fluorescent Labeling of DNA Fragments

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A method for fluorescent postlabeling of PCR products has been developed. The method uses Klenow fragment of DNA polymerase I that exchanges the 3′-terminal residue of PCR-amplified DNA fragment for fluorescent nucleotides. All reactions, including PCR, are performed in one tube simply by successive addition of reagents. The products can be applied directly to fluorescence-based automated DNA sequencers without purification for either length determination in denaturing electrophoresis or mutation detection in SSCP electrophoresis.

High-resolution electrophoresis of PCR-amplified DNA fragments is instrumental in research on genome structure, such as in linkage mapping by detecting length polymorphisms of sequence-tagged sites (Weissenbach et al. 1992) and in DNA-based diagnosis by detecting mutations within suspected genomic regions (Orita et al. 1989). Most earlier techniques in these analyses used radioactive labeling of PCR products and autoradiography to visualize separated DNA fragments. However, the demand for large-scale analysis of both target sites and DNA samples has led to the development of fluorescence-based detection of separated DNA using automated DNA sequencers (Ziegle et al. 1992; Reed et al. 1994).

Linkage analysis of human genome using fluorescence detection of length polymorphisms of simple repeat sequences has been shown to be a powerful technique in a recent study of the insulin-dependent diabetes mellitus-2 (IDDM2) gene (Pritchard et al. 1995). However, this method requires a large number of fluorescent primers for PCR amplification of the many polymorphic loci, and the cost of their chemical synthesis is considerable. Hence, a less expensive method for obtaining fluorescently labeled PCR products is preferred for further extension of this strategy to mapping of the genome in more detail, or in applying it to map genomes of other organisms.

We have been improving PCR–single strand conformation polymorphism (SSCP) analysis, which is a widely used method for detecting sequence differences in PCR product (Hayashi and Yandell 1993). In the original method, radioactively labeled PCR products amplified from control and test samples are analyzed by polyacrylamide gel electrophoresis in a single-stranded state (Orita et al. 1989; Mashiyama et al. 1990). Mutations are detected as a difference in mobility between control and test samples by autoradiography, as the test sample has a different conformation if there is a sequence change, and migrates differently in electrophoresis. PCR-SSCP requires minimal handling before separation, but the need to use a radioisotope is an obvious disadvantage.

Subsequently, fluorescence-based PCR-SSCP (F-SSCP) has been developed in which fluorescently labeled PCR products are electrophoresed and detected by automated DNA sequencer. This method is nonradioactive, highly sensitive, and has the advantage of direct data storage and processing by computer because it uses an automated sequencer. However, previously reported F-SSCP uses fluorescence-labeled primers (Makino et al. 1992), the synthesis of which is costly, and thus is not practical for large-scale analysis. A possible alternative to preparing fluorescence-labeled DNA fragments is internal labeling of the fragment with fluorescence-labeled nucleotides during PCR. However, we found that internally fluorescence-labeled PCR product showed smear on SSCP gel (Y. Kukita et al., unpubl.), probably because fluorescent nucleotides are incorporated at various positions on the PCR

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product, and the resulting labeled fragments are chemically heterogeneous.

Iwahana et al. (1995) were the first to report a method for post-PCR fluorescence-labeling that uses 3′-terminal exchange reaction by Klenow fragment of DNA polymerase I. The method is straight forward, but involves many steps including ethanol precipitation to isolate reaction products. We describe here a method for 3′-terminal postlabeling of PCR products with fluorescent dyes in which all of the steps can be performed in a single tube simply by successive additions of reagents, thus minimizing errors that might occur during sample transfer. The 3′ ends of labeled DNAs are chemically homogeneous, and several commercially available fluorescent nucleotides can be used for this purpose.

We also show that the fragments labeled by these procedures are suitable for length analysis and for conformation analysis using automated sequencers that use either slab gel or a capillary electrophoresis system (Hebenbrock et al. 1995).

RESULTS

Inhibition of 3′ Exonucleolytic Activity by 3′-terminal Fluorescent U Residues

Figure 1 shows the effects of fluorescence-labeled dUTP on the 3′ → 5′ exonuclease activity of Klenow fragment of DNA polymerase I. A 212-bp fragment that contained exon 5 of *p53* was amplified by primers PU5c and PD5a from genomic DNA of human peripheral lymphocytes. A small amount of 5′-^{32}P-labeled PU5c was also included in the PCR, therefore only the strand that extended from PU5c showed up in the autoradiogram. After PCR reaction, the amplified DNA fragment was purified and aliquots were treated with Klenow fragment in the presence of various concentrations of nucleotides.

PCR products that were not treated with Klenow fragment gave 212 nucleotides (0) and 213 nucleotides (+1) bands (Fig. 1a, lanes 1,7), the latter produced by the appending activity of *Taq* DNA polymerase (Clark 1988). After incubation with Klenow fragment without nucleotides, the two products (0,+1) disappeared and shorter fragments appeared as expected from the 3′ → 5′ exonucleolytic action of the enzyme (Fig. 1a, lane 2). Pyrimidine residues at the 3′ ends were relatively resistant to the exonuclease activity of the enzyme, as seen by the appearance of bands corresponding to T or C of the sequence ladder. The presence of increasing amounts of the four dNTPs (4–64 μM) prevented shortening, obviously because of the 5′ → 3′ polymerase activity of the enzyme (Fig. 1a, lanes 3–5). Addition of a fluorescence-labeled dUTP, R110–dUTP, had remarkable effects on the products. Although the presence of 4 μM dNTP alone was not sufficient to prevent shortening of the strand by the enzyme (Fig. 1a, lane 3), addition of 2 μM R110–dUTP blocked completely the formation of shorter fragments, and a new fragment with 3′ R110–dUMP appeared between 0 and +1 (Fig. 1a, lane 6). On the basis of these observations, we concluded that at appropriate concentrations of dNTPs and R110–dUTP, Klenow fragment removed effectively appended nucleotides and replaced 3′-terminal dTMP with R110–dUMP, but did not trim R110–dUMP from the 3′ end.

Essentially the same effects were observed with other fluorescence-labeled dUTPs (Fig. 1b),

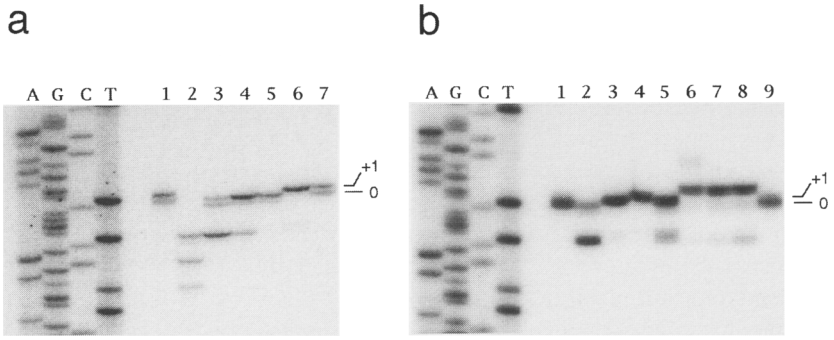

Figure 1 Effects of fluorescence-labeled dUTPs on the 3′ → 5′ exonuclease activity of Klenow fragments. (*a*) Purified PCR product in which the upper strand was labeled with 5′-^{32}P-labeled PU5c primer (lanes *1,7*), and was incubated with Klenow fragment in the absence (lane *2*) or presence of 4 μM (lane *3*), 16 μM (lane *4*), 64 μM (lane *5*) of the four dNTPs, or 4 μM of the four dNTPs and 2 μM R110-dUTP (lane *6*). Samples were resolved on a 5% polyacrylamide 8 M urea sequencing gel. Sequence ladders of the PCR product starting from 5′-^{32}P-labeled PU5c primer were also loaded in the same gel. (*b*) PCR product (lanes *1,9*) was treated with Klenow fragment and 4 μM of the four dNTP in the presence of 2 μM of dTTP (lane *2*), R110-dUTP (lane *3*), R6G-dUTP (lane *4*), TAMRA–dUTP (lane *5*), fluorescein–dUTP from Boehringer (lane *6*), fluorescein–dUTP from DuPont (lane *7*), or Fluor–dUTP (lane *8*).

although the efficiency of 3′ blockade seemed to vary depending on the fluorophore or on the mode of its coupling to the nucleotide. Blockade by R110–dUTP, R6G–dUTP, and fluorescein–dUTP from Boehringer was complete (Fig. 1b, lanes 3,4,6), whereas that by TAMRA–dUTP, Fluor–dUTP, and fluorescein–dUTP from DuPont was less efficient, at least under the present conditions. We noted the appearance of a new band of slower mobility in the samples incubated with fluorescein–dUTP from Boehringer. The band was absent in the samples incubated with other fluorescence-labeled dUTP, including fluorescein–dUTP from DuPont. Fluorescein–dUTP from Boehringer differs from others in that its fluorophore is connected to dUTP by an ethylene bridge that allows two geometric isomers, whereas the others use an acetylene bridge that does not allow any isomers (Lee et al. 1992). Thus, this slow-moving product was likely formed by a *cis*- (or *trans*-) isomer. We also noted subtle but distinct differences in mobility of the modified strand depending on the fluorophore. This effect of the fluorophore on mobility may indicate the loss of strict length dependence of the mobility of strands in sequencing gel electrophoresis if fluorescence-labeled nucleotide was incorporated internally in various numbers and positions.

Dilution of PCR Product and Postlabeling

The study described above suggested that PCR products can be labeled efficiently with fluorescence simply by diluting and adding appropriate reagents. We used fluorescein–dUTP from Boehringer to estimate labeling efficiency because the labeled product shows a clearly distinguishable mobility shift (Fig. 1b). PCR amplification was carried out including 5′-^{32}P-labeled PU5c primer. The amplification product was diluted two- to six-fold and fluorescence labeling reactions were allowed to proceed by adding fluorescein-dUTP and Klenow fragment and incubating at 37°C for 15 min. Samples were then analyzed by sequencing gel electrophoresis (Fig. 2). We found that most of the radioactivity shifted to the position of the fluorescein-labeled product at all dilutions, indicating that postlabeling by fluorescent–dUTP is efficient in diluted PCR reaction mixture that contains dTTP in a range of 33 μM (85% label at sixfold dilution) to 100 μM (60% label at twofold dilution).

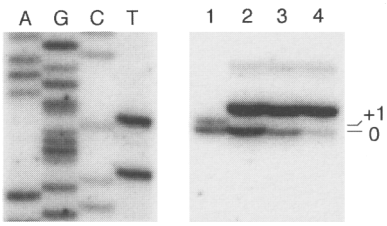

Figure 2 Direct fluorescence-labeling of diluted PCR mixture. PCR reaction mixture containing amplified product between primers 5′-^{32}P-labeled PU5c and PD5a was diluted twofold (lane *2*), fourfold (lane *3*), or sixfold (lane *4*), and incubated with 0.1 U/μl of Klenow fragment in the presence of 2 μM fluorescein–dUTP (Boehringer) at 37°C for 15 min. Untreated PCR product was loaded on lane *1*. Fractions of fluorescence-labeled PCR products, which migrated slower than nontreated products, were ~60% (lane *2*), 70% (lane *3*), and 85% (lane *4*), as measured by an image analyzer.

Fluorescence Detection in Polyacrylamide Slab Gel Electrophoresis

Figure 3 shows that the fragments labeled with fluorescein–dUTP by the above procedures can be analyzed directly with an automated DNA sequencer that separates fragments in polyacrylamide slab gel, either for length determination or for conformation analysis. Exons 5, 7, and 8 of *p53* were PCR amplified using primers with A at their 5′ ends, and postlabeled with fluorescein–dUTP after fourfold dilution. The samples were then diluted with formamide solution, heat-denatured and subjected to electrophoresis either under denaturing conditions (Fig. 3a) or SSCP conditions (Fig. 3b) using an ALF automated DNA sequencer. All of the labeled PCR products showed single sharp peaks under denaturing conditions (Fig. 3a), which were split into two peaks of complementary single strands under SSCP conditions (Fig. 3b), demonstrating that both strands were labeled by the present method. Under SSCP conditions, the two peaks of the complementary strands were not the same height suggesting that the efficiency of labeling differed between the fragments. This difference was reproducible and did not hamper mutation detection by SSCP analysis (data not shown).

The unincorporated fluorescein–dUTP migrated in peaks in the range of short fragments and did not interfere with product peaks under either denaturing or SSCP conditions. Different results were obtained when R110–dUTP or R6G–

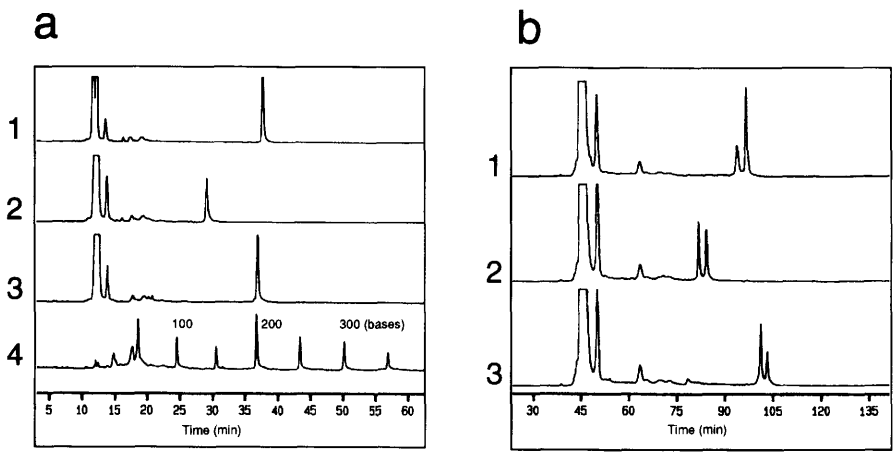

Figure 3 Polyacrylamide slab gel electrophoresis of fluorescence-labeled PCR product. PCR products of exons 5, 7, and 8 of the *p53* gene amplified from THP-1 cells were labeled by fluorescein–dUTP (Boehringer). Samples were heat-denatured and electrophoresed in an ALF automated DNA sequencer in denaturing 6% Long-Ranger gel with a separation distance of 9 cm (*a*), or in SSCP gel with a separation distance of 22 cm (*b*). Lanes *1–3* show PCR products of exons 5, 7, and 8, respectively. Sizer 50–500 (Pharmacia Biotec.) was applied to lane *4* (*a*) as a size standard.

dUTP was used for labeling. These nucleotides migrated in several peaks, and migrated to the 0- to 150-base range under both denaturing and SSCP conditions (data not shown). The cause of the apparent heterogeneity of these nucleotides in electrophoresis is unknown. We found that treatment of the labeling mixture with calf intestine alkaline phosphatase in the presence of EDTA effectively eliminated these peaks (data not shown), presumably by removing the negative charges of the nucleotides, as discussed later.

Capillary Electrophoresis

Figure 4 shows an example in which *p53* exon 8 was PCR amplified, labeled with R6G–dUTP and analyzed by a Prism 310 Genetic Analyzer under denaturing conditions. As shown, unincorporated R6G–dUTP migrated in three peaks, at around the 100- to 250-base region, that overlapped the region to which the peak of the PCR product migrated (Fig. 4a, b). The nucleotide peaks were completely eliminated by treatment with calf intestine alkaline phosphatase (Fig. 4c). Unincorporated R110–dUTP gave results similar to those with R6G–dUTP in capillary electrophoresis, that is, it migrated to around the 90- to 150-base region, and could be eliminated by alkaline phosphatase treatment (data not shown).

SSCP Analysis of Postlabeled PCR Product by Capillary Electrophoresis

The efficacy of the present labeling method in detecting mutation by SSCP analysis was also tested using capillary electrophoresis. Exon 8 of the *p53* gene was amplified from genomic DNA of THP-1, Namalwa and Daudi cells, and used for labeling with R6G–dUTP after fourfold dilution. Samples were then treated with calf intestine alkaline phosphatase, heat-denatured in formamide, and separated by capillary electrophoresis under SSCP conditions. We have found that THP-1 is homozygous for the normal allele of *p53* exon 8, Daudi is heterozygous for the normal and G266E (GGA → GAA) mutant allele, and Namalwa is heterozygous for the normal and R282W (CGG → TGG) allele (Y. Kukita et al., unpubl.). Figure 5 demonstrates that the three alleles can be distinguished clearly by the distinct mobilities of their rapidly migrating bands in the present system.

DISCUSSION

We developed a single tube method for fluorescent postlabeling of PCR product. The method takes advantages of the fact that fluorescence-labeled dUTP is incorporated efficiently into the 3′ end of double-stranded DNA that ends with T even in the presence of excess dTTP, and that the 3′ fluorescence-labeled nucleotide blocks exonuclease activity of Klenow fragment. The method described here has several advantages over existing fluorescence-labeling techniques of PCR products. First, PCR can be performed with an unmodified primer, thus saving cost and time for the chemical synthesis of fluorescence-labeled primers. However, this cost-saving may be counterbalanced partly by the use of fluorescent nucleotides and additional enzymes in each reaction. Second, there is no transfer of samples to

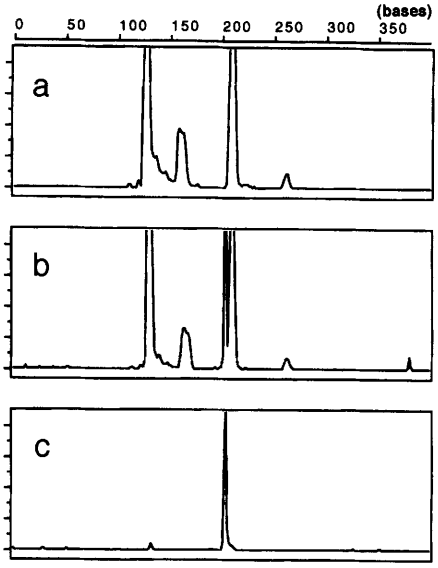

Figure 4 Capillary electrophoresis of PCR products postlabeled and treated with calf intestine alkaline phosphatase. Electropherogram of free R6G–dUTP (*a*), PCR product between PU8a–PD8A postlabeled with R6G–dUTP (*b*), and PCR product postlabeled and treated with calf intestine alkaline phosphatase (*c*). Electrophoresis was performed under denaturing conditions at 42°C.

other tubes, which simplifies handling and minimizes the chance of making trivial errors. This second feature is in contrast to a previously reported postlabeling method (Iwahana et al. 1995) that involves purification steps before and after the labeling reactions to remove unincorporated nucleotides.

Another advantage of the present method is that 3′ ends of the labeled products are homogeneous because the 3′ extra nucleotides attached by the appending activity of *Taq* DNA polymerase are removed during the labeling reaction by Klenow fragments. It has been reported that the presence of appended molecules perturbs fragment-length analysis in some cases (Smith et al. 1995).

We found that treatment with phosphatase was effective in eliminating peaks of unincorporated R110–dUTP and R6G–dUTP, both of which carry rhodamine-derived fluorophores. R110–ddNTP and R6G–ddNTP are used routinely as dye terminators for dideoxy sequencing using an Applied Biosystems, Inc. (ABI), automated sequencer. It has been suggested that unincorporated rhodamine dye terminators should be removed thoroughly from the reaction mixture by ethanol precipitation, as they interfere with the sequencing ladder (Lee et al. 1992). We believe that treatment with phosphatase is a more convenient method for removing interfering peaks of free rhodamine nucleotide in sequencing electropherogram, and also in other experiments using internal fluorescence-labeling procedures. However, treatment of unincorporated fluorescein–dUTP with phosphatase is not recommended because dephosphorylated product migrates at around the 100-base region in electrophoresis and is likely to disturb most of the analysis (data not shown). The difference in mobility of fluorescein- and rhodamine-labeled nucleotides is attributable to the difference in the negative charges of the fluorophores (Lee et al. 1992).

Although the method worked for the target sequences described here, if the same is also true for any other sequence contexts remains to be tested. In particular, when terminal sequences are thermally unstable, such as stretches of AT pairs, Klenow fragment may then remove more than one nucleotide and fluorescent dUTP may be incorporated at internal positions. Therefore, it should be safe to choose primers that are devoid of A's in at least, for example, four nucleotides to their 5′ ends except for extreme termini.

The method we developed should be particularly beneficial in large-scale analyses that use PCR and high-resolution electrophoresis, such as linkage mapping using length polymorphisms of

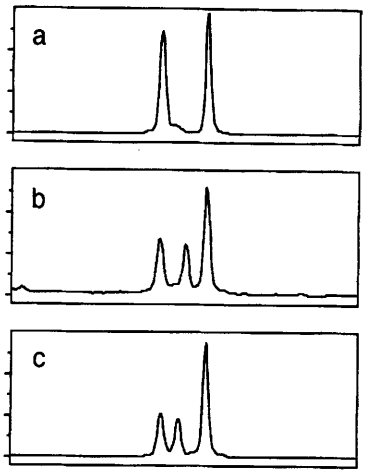

Figure 5 SSCP analysis of postlabeled PCR products of *p53* exon 8 in capillary electrophoresis. Exon 8 of *p53* was amplified by PCR from genomic DNA of THP-1 (*a*), Namalwa (*b*), and Daudi (*c*) cells, postlabeled with R6G–dUTP, treated with phosphatase, and analyzed by capillary electrophoresis under SSCP conditions at 30°C.

simple repeats (Reed et al. 1994), sequence-tagged site (STS) content mapping of yeast artificial chromosome (YAC) clones (Chumakov et al. 1992), or mutation detection in clinical samples by SSCP analysis for DNA diagnosis (Orita et al. 1989).

METHODS

Reagents

R6G–dUTP, R110–dUTP, and TAMRA–dUTP were obtained from Applied Biosystems Division/Perkin-Elmer (Foster City, CA). Fluorescein–dUTP was from Boehringer Mannheim (Penzzberg, Germany) or DuPont NEN (Boston, MA). Fluor–dUTP was from Stratagene (La Jolla, CA). Klenow fragment of DNA polymerase I and polynucleotide kinase were obtained from New England Biolabs (Beverly, MA). Calf intestine alkaline phosphatase was obtained from Boehringer Mannheim. All other reagents were as suggested by the instrument manufacturers or of the highest quality available. DNAs were extracted from Burkitt lymphoma cell lines (Daudi, Namalwa), a monocytic leukemia cell line (THP-1), and human peripheral blood lymphocytes following standard procedures (Sambrook et al. 1989).

PCR

The PCR primers for the human *p53* gene were designed according to the reported sequence (EMBL accession no. X54156). An extra A was added to the 5′ ends of some of the primers so that the 3′ends of PCR products would end with T (Table 1). PCR amplification was performed in 5–50 μl of reaction mixture that contained 0.4 μM of each of the two primers, 0.2 mM of each of the four nucleotides, 4 ng/μl of template DNA, 0.04 U/μl of *Taq* DNA polymerase (Perkin-Elmer, Norwalk, CT), 8.8 ng/μl of *Taq*Start antibody (Clontech, Palo Alto, CA), 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), and 50 mM KCl. Thermal cycling was carried out in a Perkin-Elmer 2400 cycler. The cycling profile was 1 min at 95°C for the initial heating, followed by 35 cycles of 30 sec at 95°C for denaturation, 45 sec at 66°C (exons 5 and 7) or 55°C (exon 8) for annealing, 90 sec at 72°C for extension, and a final incubation at 72°C for 7 min. When necessary, aliquots of PCR products were electrophoresed on 2% agarose gels, and the amounts of amplified fragments were estimated by the intensities of bands stained with ethidium bromide. A known amount of *Hin*fI-digested pGEM-2 (Promega, Madison, WI) was separated in the same gel, and served as a standard for size and quantity.

Fluorescent Labeling of Purified Radioactive PCR Products

The PU5c primer was labeled at its 5′ end by polynucleotide kinase and [γ-^{32}P]ATP (ICN, Irvine, CA) according to the method previously described (Mashiyama et al. 1990). A small amount of the labeled primer was added to a PCR mixture that contained primers PD5a and PU5c to amplify exon 5 of the *p53* gene in DNA of human peripheral blood leukocytes. The PCR product was purified with Wizard PCR Preps (Promega, Madison, WI) and 0.2-pmole aliquots were treated with 2 units of Klenow fragment at indicated concentrations of nucleotides in 10 μl of 10 mM Tris-HCl (pH 8.5), 5 mM $MgCl_2$, and 7.5 mM DTT at 37°C for 30 min. The reactions were stopped by adding 20 μl of 95% formamide, 10 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol. Samples were then heat-denatured and separated by 5% acrylamide gel containing 8 M urea. A sequencing ladder starting from the 5′-^{32}P-labeled PU5c primer was used as a size marker. Gels were dried and exposed to X-ray film (New RX: Fuji Film, Tokyo) or to an imaging plate and the radioactivity of each band was measured using a BAS2000 Bio-imaging Analyzer (Fuji Film, Tokyo).

Direct Fluorescent Labeling of PCR Products

Typically, 5 μl of the PCR product was used for labeling reaction with fluorescence-labeled dUTP after fourfold dilution and bringing to 10 mM Tris-HCl (pH 8.7), 10 mM $MgCl_2$, and 2 μM fluorescein–dUTP. Labeling reactions were performed by adding 0.1 U/μl of Klenow fragment and incubating the mixture at 37°C for 15 min. When R110–UTP or R6G–UTP was used for the label, 2 μl of 0.2 M EDTA and 2 μl of 1 U/μl of calf intestine alkaline phosphatase were added and the mixture was incubated at 37°C for 30 min. The reaction was stopped by adding 80 μl of formamide.

Electrophoresis in Automated DNA Sequencers

Slab gel electrophoresis of fluorescence-labeled PCR product was carried out using an ALF automated DNA sequencer (Pharmacia Biotec, Piscataway, NJ) with a built-in water jacket connected to an external thermostat-regulated water circulator. The mixture of labeling reaction was brought to 20 mM EDTA by adding concentrated solution and diluted with 10 volumes of formamide con-

Table 1. Primers to Amplify Various Exons of the *p53* Gene

Exon	Primer	Nucleotide sequence	Amplicon (bp)
5	PU5c	CTCTTCCTGCAGTACTCCCCTGC	212
	PU5A	ACTCTTCCTGCAGTACTCCCCTGC	213
	PD5a	AGCCCCAGCTGCTCACCATCGCTA	
7	PU7A	AGTGTTGTCTCCTAGGTTGGCTCTG	141
	PD7A	ACAAGTGGCTCCTGACCTGGAGTC	
8	PU8a	ACCTGATTTCCTTACTGCCTCTTGC	201
	PD8A	AGTCCTGCTTGCTTACCTCGCTTAGT	

An extra A residue is underlined in each nucleotide sequence.

taining 5% blue dextran. The diluted mixture was heated at 90°C for 5 min and loaded to the gel at 1–3 μl per lane. Denaturing electrophoresis was performed in 6% Long-Ranger gel (AT Biochem, Malvern, PA) containing 7 M urea, following the supplier's instructions. SSCP analysis was performed as previously described (Makino et al. 1992). The temperature of the water circulator was 47°C for denaturing conditions and 25°C for SSCP conditions. The results were analyzed by Fragment Manager software (Pharmacia Biotec).

Capillary electrophoresis using a replaceable polymer matrix was performed with an ABI Prism 310 Genetic Analyzer (Applied Biosystems Division/Perkin-Elmer, Foster City, CA). An aliquot (0.5–1 μl) of labeled product in formamide was transferred to a sample tube for Prism 310 that contained 1 μl of GeneScan-500 TAMRA size standard, 1 μl 0.1 N NaOH, 1 μl 30 mM EDTA, 24 μl of formamide, and 2 μl H_2O. The conditions for capillary electrophoresis were based on the manufacturer's instructions. The effective capillary length (the length to the detector) was 30 cm. For denaturing electrophoresis, Genetic Analyzer Buffer containing 2.8% GeneScan polymer and 8 M urea was used for the cathode side and buffer containing 2% GeneScan polymer was used for the anode side. For SSCP analysis, 1× TBE buffer containing 3% GeneScan polymer and 10% glycerol was used for both sides. The samples were heated at 90°C for 5 min, and electrophoretically loaded to the capillary at 7 kV for either 5 sec (denaturing gel electrophoresis) or 20 sec (SSCP). The electrical field during the run was 317 V/cm. The results were analyzed by GeneScan software (Applied Biosystems Division/Perkin-Elmer).

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