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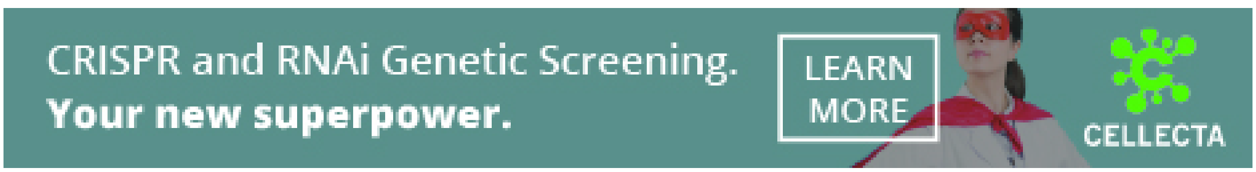

A Nonisotopic Single-strand Conformation Polymorphism Protocol Using a Direct Blotting Electrophoresis, a Chemiluminescent Detection System, and a Multiplex Approach

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Besides ribonuclease cleavage,(1) denaturating gel electrophoresis,(2–5) chemical cleavage,(6) and heteroduplex analysis,(7,8) single strand conformation polymorphism (SSCP) has become a simple and sensitive method for the rapid detection of mutations on a gene level.(9–11) This method is based on the principle that the electrophoretic mobility of single-stranded DNA molecules in nondenaturing polyacrylamide gels not only depends on their size but also on their sequence. Single base exchanges and minor deletions or insertions in a given sequence of genomic DNA form different conformers and therefore migrate differently during electrophoresis. SSCP cannot reveal the nature of the mutation, as any sequence variation might change the pattern of migration of single-stranded DNA. The exact type of mutation still has to be determined by direct sequencing. By use of this approach SSCP has been shown to be highly sensitive in detecting mutations in the genes for hemophilia B,(12) neurofibromatosis type II,(13) osteogenesis imperfecta type II,(14) and familial hypercholesterolemia (FH).(15,16) Additionally, SSCP was used successfully to detect mutations in the *ras* oncogene,(17) the p53 tumor suppressor gene,(18) and the retinoblastoma gene.(19)

The use of SSCP according to conventional protocols(9–11) is limited by the need for radioactive labeling of PCR products and time-intensive electrophoresis of large gels sized like sequencing gels. Smaller gels have also been used and may be easier to handle; and in these gels DNA fragments may be detected in a nonradioactive manner by silver staining.(20) However, small, thick gels are limited in their resolution capacity and therefore may be less sensitive. As the optimal fragment size for sensitive detection of base substitution is ~150 bp,(21) large genes must be split into many fragments, that is, each exon must be analyzed separately.

The aim of this study was to develop a nonradioactive SSCP protocol that overcomes most of these restrictions but results in a resolution capacity and sensitivity comparable to standard radioactive SSCP protocols in DNA fragments containing as little as a single base substitution. This was demonstrated for known mutations in exon 14 and exon 10 of the low density lipoprotein (LDL) receptor gene, which had been identified previously by use of large gels for separation of radioactively labeled PCR fragments in the SSCP analysis and subsequent direct sequencing of the fragments with a suspected sequence variation (H. Knoblauch et al., unpubl.).

The nonisotopic SSCP protocol described here uses a direct DNA blotting electrophoresis system, a novel vinyl polymer-based acrylamide gel matrix instead of standard polyacrylamide and a chemiluminescent detection system of biotinylated DNA fragments instead of radiolabeled PCR fragments. Furthermore, by use of this procedure several DNA fragments can be analyzed during the same gel run.

MATERIALS AND METHODS

PCR

Total genomic DNA was extracted from white blood cells of 10 patients with clinically suspected FH using a Triton X-100 lysis method.(22) Exon 10 and exon 14 of the LDL receptor gene were amplified using PCR(23) and oppositely oriented 25-base oligonucleotides complementary to intron sequences that flank the exons as described previously.(16) The oligonucleotides were synthesized using an ABI 391 DNA synthesizer (Applied Biosystems, Foster City, CA), and one of the oligonucleotides was biotinylated on its 5′ end during synthesis using β-cyanoethyl-*N*,-*N*-diisopropyl-phosphoramidite. The PCR reaction was performed in a total volume of 50 μl, containing 0.2 μg of genomic DNA, 30 pmoles of each primer, 30 nmoles of each dNTP, and 1.25 units of DNA polymerase *Thermus aquaticus* (Ampli*Taq*, Perkin-Elmer Cetus, Norwalk, CT) in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001% gelatin. Each PCR reaction was overlaid with mineral oil and subjected to one cycle at 95°C for 5 min and 68°C for 2 min followed by 30 cycles at 95°C for 1 min and 68°C for 2 min (Perkin-Elmer Cetus thermocycler).

Electrophoresis and Chemiluminescent Detection

A 24×36×0.02-cm gel was prepared from 0.5 X Hydrolink MDE (mutation detection enhancement) matrix (MDE, AT Biochem, distributed by Serva Feinbiochemica, Heidelberg, Germany). In a previous study, Hydrolink MDE gel, a vi-

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nyl polymer based on acrylamide, was shown to improve the resolution efficiency in the detection of single-stranded DNA in SSCP and double-stranded DNA duplexes in heteroduplex analysis.[24] The PCR product (3 μl) was mixed with 5 μl of formamide dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), boiled at 95°C for 3 min to denature the double-stranded DNA to single strands, and snap-cooled on ice. An aliquot of 0.5 μl was loaded on the gel. The electrophoresis was performed using the TE 2000 Direct Blotter (Hoefer, San Fernando, CA, distributed by Serva Feinbiochemica). This apparatus allows direct blotting of DNA fragments during the gel run onto a nitrocellulose membrane (Pall, Biodyne A, Serva Feinbiochemica) that is pulled under the bottom of the plates at a constant speed of 5 cm/hr. Electrophoresis conditions were 6 W at room temperature for 18 hr. The run of the membrane was started 8 hr after the gel was loaded. After electrophoresis and transfer of DNA onto the membrane, the fragments were UV cross-linked to the membrane. Detection of the biotin-labeled DNA was carried out using the CSPD chemiluminescent detection system [CSPD = disodium 3-(4-methoxyspirol[1,2-dioxetane-3,2′-(5′chloro)tricylo[3.3.1.13,7] decan]-4yl)phenyl phosphate] following the manual of the detection kit (SeqLight Kit, Serva Feinbiochemica), using CSPD as the substrate for the alkaline phosphatase reaction. Finally, the membrane was exposed to an x-ray film for 2 hr.

RESULTS AND DISCUSSION

SSCP analysis of exon 14 of the LDL receptor gene in 10 different DNA samples using the standard protocol showed a different pattern of migration in 3 of the 10 fragments (Fig. 1A). Direct sequencing of the respective exon of the LDL receptor gene revealed that the patients' DNA fragments analyzed in lanes 1 and 3, which showed an identical pattern of bands in the SSCP analysis, carried an identical point mutation at codon 660 resulting in a TGC → TAC base substition and a cysteine → tyrosine amino acid substitution. The patient analyzed in lane 6 carries a CCG → CTG base substitution in codon 678 that results in a proline → leucine amino acid exchange (unpubl.). The same DNA samples were then used to compare these results with the newly described SSCP protocol using the TE 2000 direct DNA blotting system and the chemiluminescent detection method of biotin-labeled PCR fragments after blotting the DNA onto a nylone membrane. Figure 1B shows that the pattern of the band is identical. Although the bands are slightly less sharp, probably caused by the blotting process, there is no loss in sensitivity with the new protocol.

In a further experiment, exons 10 and 14 of the LDL receptor gene from 10 patients were amplified separately by PCR but were loaded simultaneously into one slot of the gel and subject to electrophoresis under the same conditions as described above (Fig. 2). For this experiment the gel from the previous experiment was used. Fragments from the two exons were clearly resolved by their different mobility, although both fragments were loaded at the same time. A mobility shift was observed in lanes 2, 5,

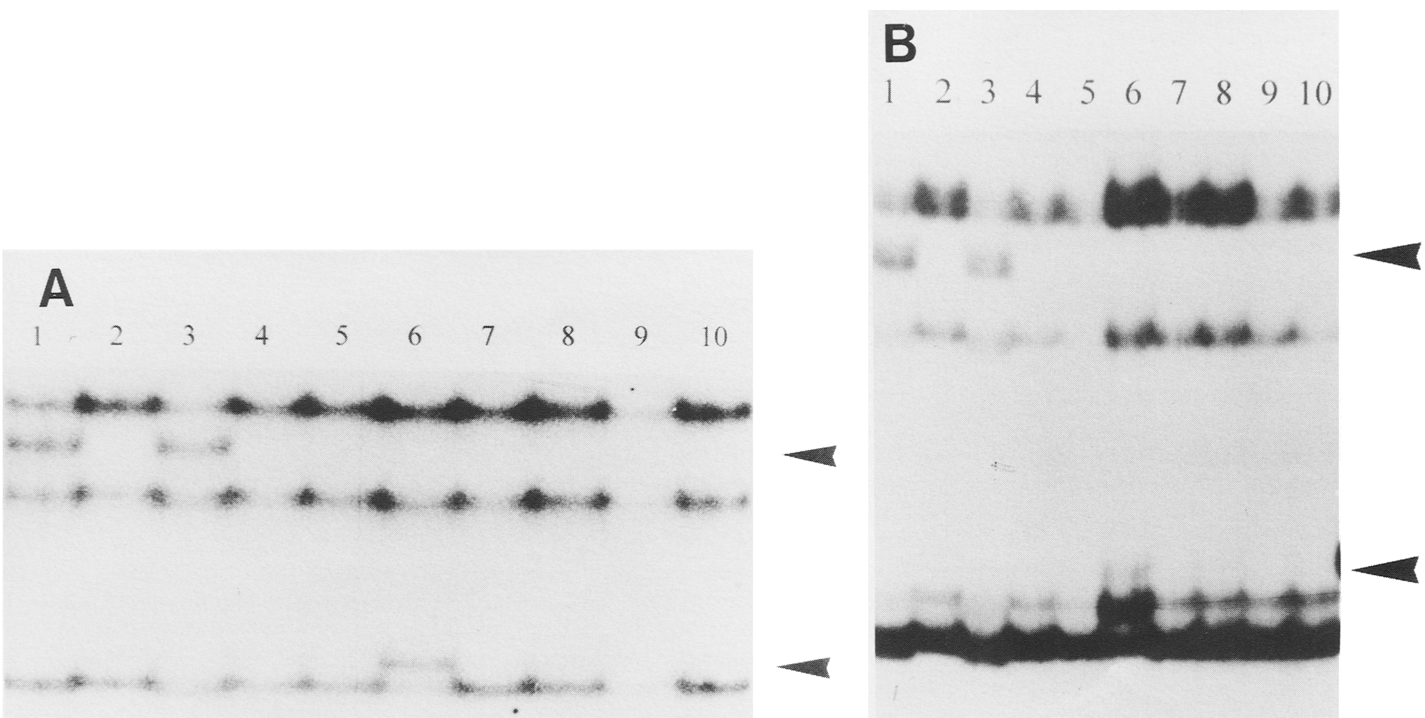

FIGURE 1 (*A*) SSCP analysis of exon 14 of the LDL receptor gene in 10 different DNA samples using ^{32}P-labeled PCR products according to the standard protocol.[17] The DNA fragments in lanes *1*, *3*, and *6* show an additional band (arrowhead), indicating sequence variations. The bands in lane *9* are missing because of a PCR dropout. (*B*) SSCP analysis of exon 14 of the LDL receptor gene in the same DNA samples as in *A*. The newly described SSCP protocol uses the TE 2000 direct DNA blotting system and chemiluminescent detection method of biotinylated PCR fragments after blotting the fragments onto a nylone membrane.

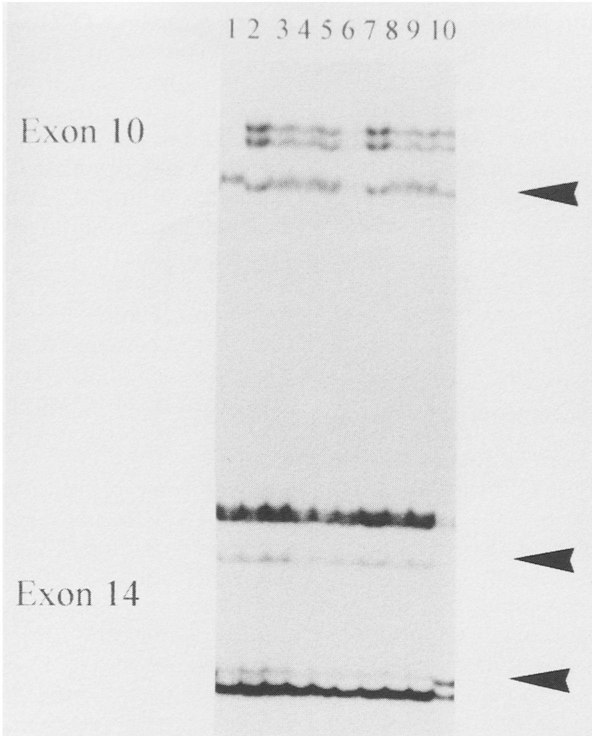

FIGURE 2 Multiplex SSCP analysis of exons 10 and 14 of the LDL receptor gene in 10 different DNA samples. Fragments from the two exons are clearly separated by their different mobility. A mobility shift is observed in lanes *2*, *5*, *7*, and *10* at the bands of exon *10*, indicating the known Bsm AI polymorphism and in lane *1* of the bands of exon 10 (arrowhead) and in lane 10 of the bands of exon 14 (arrowhead), indicating unknown variations in the sequence, which still need to be confirmed by direct sequencing. The missing bands of exon 10 in lane *6* are caused by PCR dropout.

7, and 10 at the bands of exon 10 indicating the Bsm AI polymorphism that is caused by a nucleotide change in codon 450 (AGA → AGG), which does not result in an amino acid exchange. Additionally, a different pattern of bands was observed in lane 1 at the bands of exon 10 and at lane 10 at the bands of exon 14, indicating unknown variations in the sequences of the corresponding exons that still need to be confirmed by direct sequencing.

We have shown that the results of this protocol are similar to conventional protocols using high-resolution gels sized like sequencing gels and ^{32}P-labeling of PCR products in terms of resolution capacity and sensitivity. Additionally, we have demonstrated that a multiplex SSCP analysis of two exons is feasible without loss of sensitivity. Furthermore, because of the blotting device the high-resolution electrophoresis gel based on the MDE gel matrix can be reused.

This protocol has further advantages. There is no need to use hazardous radioactivity. The exposure time of the gel using chemiluminescent detection is in the range of 1–3 hr compared with the usual 24 hr using a radioactive label. Because of the principle of the blotting device, the maximum length of the gel is used for resolution of SSCP. Several samples can be loaded at the same time in the same slot. The same gel can be reused several times because there is no need for drying the gel or taking it out of the gel cast for capillary blotting. Experiments where more than two exons of the LDL receptor gene are amplified at the same time and analyzed by SSCP are under way, which will also accelerate the screening procedure. Therefore, the multiplex approach and the reusability of gels compensates for the disadvantage of the time-consuming preparation of thin, large gels and the need for nylon membranes in comparison with conventional SSCP protocols.

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