Detection of Unknown Gene Mutations by Multiplex Single-strand Conformation Polymorphism (MSSCP)

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Detecting unknown mutations is a problem common to most genes, because of the large degree of heterogeneity of DNA variations leading to the expression of a mutant phenotype. The issue is particularly relevant in humans, where it is very important to pinpoint single mutations producing inherited diseases, not only for practical purposes such as prenatal or presymptomatic diagnosis, but also for the study of possible correlations between the kind of mutation and the clinical phenotype.

Although different methods were devised for this purpose, such as chemical cleavage,⁽¹⁾ denaturing gradient gel electrophoresis (DGGE),⁽²⁾ and temperature gradient gel electrophoresis (TGGE),⁽³⁾ single-strand conformation polymorphism (SSCP)⁽⁴⁾ is perhaps the most popular method described to date. The rationale of the method is that single-stranded DNA sequences differing by a single-base change may show different electrophoretic mobility in a polyacrylamide gel because of conformational change produced by the substitution.⁽⁴⁾

Multiplex PCR amplification was originally devised for detection of exon deletions⁽⁵⁾ in the human dystrophin gene. A modification of this method, using polyacrylamide gel electrophoresis, was later introduced, allowing the detection of intraexonic deletions or duplications.⁽⁶⁾

A first attempt at developing multiplex SSCP was reported recently. The investigators succeeded in detecting 4 base substitutions by radioactive multiplex PCR amplification, followed by gel electrophoresis of the denatured amplification products.⁽⁷⁾ The size of the amplification products in which base substitutions were detected ranged from 238 to 547 bp.

A further refinement of this approach is reported here. To obtain sharper resolution, the present method implies the labeling of all forward primers involved in the multiplex PCR amplification, instead of using labeled dNTP.

MATERIALS AND METHODS

DNA samples were obtained either by phenol/chloroform extraction or by salting-out, from peripheral blood samples, according to standard procedures. The

control DNA was from healthy males, whereas the mutated DNAs were from patients affected by Duchenne muscular dystrophy, in whom base substitutions were characterized previously by DNA sequencing.⁽⁸⁾

Forward primers (MedProbe) for the amplification of different exons of the dystrophin gene were 5'-end-labeled with [γ -³²P]ATP, by using T4 polynucleotide kinase (Promega).

In a final volume of 10 μ l, 10 pmoles of dephosphorylated forward primers were incubated for 30 min at 37°C in the presence of 5 pmoles of [γ -³²P]ATP [3000 Ci/mole; 10 μ Ci/ μ l (Amersham)] in appropriate buffer [0.5 M Tris-HCl (pH 8.0), 0.1 M MgCl₂, 50 mM DTT, 1 mM spermidine, 1 mM EDTA].

After incubation, the kinase was inactivated by heating at 90°C for 2 min. The solution was then stored at -20°C before use for as long as 1 month. In a standard multiplex PCR, 200 ng of human genomic DNA was added to 25 μ l of mixture [1.5 mM MgCl₂, 67 mM Tris-HCl (pH 8.8), 16.6 mM ammonium sulfate, 0.001% Tween 20, 200 μ M of each dNTP, 4.25 pmoles of each reverse primer, 0.5 pmoles of each labeled forward primer, 2.5 pmoles of each nonlabeled forward primer, and 2 units of recombinant thermostable DNA polymerase RTB (Bioline, Italy)]. The cycles (3 min at 94°C, followed by 25 steps of 30 sec at 94°C, and 2 min at 65°C) were performed by a PTC-100 MJ Research, Inc., Thermocycler. Vertical slab electrophoreses (40 $\times$ 20 cm, 0.04 cm thick) were run on 8% polyacrylamide gel (29:1 acrylamide/bis-acrylamide) added with 10% glycerol, at room temperature, using 1 $\times$ TBE buffer (45 mM Tris-borate at pH 8.3; 4 mM EDTA) at 25 watts for 6 hr. A metal plate was attached to the front plate of the vertical gel apparatus to ensure thermal homogeneity.

Just before loading the samples, 4 μ l of the amplification product was denatured by adding 4 μ l of denaturing loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). The solution was heated (94°C) for 2 min and then chilled immediately on ice.

After electrophoresis, the gels remained for 1 hr in a Bio-Rad gel dryer (model 583). Once desiccated, they were autoradiographed (Hyperfilm-MP, Amersham) by a 16-hr exposure at room temperature.

RESULTS AND DISCUSSION

An example of multiplex SSCP is reported in Figure 1: One set of 10 different amplification products was separated by polyacrylamide gel electrophoresis under the conditions described above. In this case, the size of the amplification products ranged from 100 to 547 bp. Shifts in the electrophoretic mobility of

FIGURE 1. Autoradiographs obtained from multiplex SSCP. Ten different exons of the dystrophin gene were amplified in a multiplex PCR reaction in which forward primers were radioactively labeled. The mobility shifts are indicated (arrows). The number of different exons is specified at left. (Lanes A,D) DNA from a male control subject; (lane B) patient 620 (A → T substitution in the amplification product of exon 48); and (lane C) DNA from patient 535 (C → T substitution in exon 41).

DNA fragments are clearly visible. In this case, the base substitutions were detected in sequences of 275 and 506 bp. Therefore, this method would enable us to detect base substitutions in amplification products as large as 500 bp. The possibility of detecting base changes in sequences cannot be excluded, because a mutation was detected by SSCP in an amplification product that was 547 bp long.⁽⁷⁾

The sensitivity of the method might possibly be improved by controlling the temperature of the gel matrix. In the experimental conditions described in this paper, the temperature of the gel matrix was ~30°C, which proved to be optimal, empirically. A poorer resolution was obtained with lower temperatures (~20°C).

According to present knowledge, the probability of detecting a point mutation as a single strand electrophoretic mobility shift was estimated to be 99% for fragments up to 300 bp and ~90% for fragments from 300 to 450 bp.⁽⁹⁾ Methods alternative to SSCP for detecting point mutations are presently less sensitive or technically more complicated. For instance, chemical cleavage, which is the most powerful method described so far, involves complicated protocols and the use of hazardous chemicals.

The present method is very simple and the single-stranded labeling of the amplification products strongly ameliorates the resolution of electrophoretic bands. The number of single amplification products included in the multiplex amplification might even be increased. By choosing appropriate sets of multiplex PCR, large segments of a given gene may be easily screened to detect unknown mutations. As far as the dystrophin gene is concerned, the present method enables us to perform simultaneously the screening for exonic deletions, for intraexonic deletions, additions, or duplications, and for a number of base substitutions.

Despite the size of the coding sequence (14 kb) and the large number of exons to be screened (79), only eight sets of multiplex PCR might enable us to detect the large majority of mutations in any patient affected with Duchenne or Becker muscular dystrophy.

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NOTE ADDED IN PROOF

The possible reassociation and the formation of double-stranded molecules can be prevented by increasing the concentration of formamide in the loading buffer and by working at very low concentrations of DNA. The visualization of SSCP is enhanced by strongly reducing the concentration of the unlabeled molecules of the forward primers.

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