Mutation detection is a key step for the identification of genes in inherited diseases. One of the most widely used techniques for detection of unknown mutations is single-strand conformation polymorphism (SSCP) analysis. SSCP analysis is based on the principle that a single-stranded DNA molecule has its own specific sequence-based secondary structures in a non-denaturing gel matrix. A DNA fragment with a single base modification may form different conformers and migrate differently under non-denaturing conditions. The original SSCP protocol used the incorporation of radioactive labels for detection (10). Other nonradioactive polymerase chain reaction (PCR)-SSCP protocols, using silver staining (8) and fluorescence labeling (2,6,7), have also been described.

We used the PRISM™ 377 Automated DNA Sequencer (PE Applied Biosystems, Foster City, CA, USA) and the sensitivity of fluorescence analysis for mutation screening with a newly developed fluorescence-based SSCP protocol. This protocol was evaluated by screening for five known mutations in the glucokinase (GCK) gene (3,4), two...
recently described mutations in the human Kir6.2 gene, a subunit of the beta-cell ATP-sensitive K channel (11) and three other known mutations in the sulfonylurea receptor (SUR) gene (5). The GCK gene was the first gene shown to be responsible for a subtype of non-insulin-dependent diabetes mellitus (NIDDM) called maturity-onset diabetes of the young (MODY) (3). This gene was chosen for two reasons. The first is that the availability of a large number of different mutations (over 100 have been reported all over the world). Second, routine screening of the intronless gene Kir6.2.

In the course of a research program on genetic determinants of obesity, the search for mutations in the human mitochondrial uncoupling protein (UCP) gene was initiated. The UCP gene was investigated on the basis of an association between a high weight gain in obese patients and the 8.3-kb allele of a Bc/I polymorphism 3 kb upstream of the gene (9). The method described in this report was applied to screen the promoter sequence of the human mitochondrial UCP gene (1) for the presence of new mutations in a population of 227 massively obese patients (body mass index [BMI] > 40 kg/m²) and 99 nonobese subjects (BMI < 27 kg/m²).

For all the different mutations studied, genomic DNA was amplified on a PTC-200™ Thermal Cycler (MJ Research, Watertown, MA, USA) using each DNA fragment’s specific oligonucleotides extended by the universal -21 M13 (TGTTAAACGACGAGTCT) and M13 Reverse (CAGGAAACAGCCTCCGGTTG) sequences at their 5’ ends. Amplifications were carried out in a total volume of 50 μL with 100 ng of genomic DNA, 1 U of Taq DNA Polymerase (Perkin-Elmer, Norwalk, CT, USA) and 0.25 μM of each primer. PCR cycling conditions for the GCK, Kir6.2 and SUR genes have been previously described (7–9). Primers M13 Reverse UCP2 (5’ CAGGAAACAGCCTCCGGTTG) and -21 M13 UCP5 (5’ TGTAAACGACGAGTCT) previously reported through radioactive and/or silver-staining SSCP methods (Table 1). For the GCK gene, 5 mutations in Exons 3, 7 and 9 were detected in families with MODY, where these mutations were originally described. In other patients with late-onset diabetes of the young (MODY) (3). This gene was chosen for two reasons. The first is that the availability of a large number of different mutations (over 100 have been reported all over the world). Second, routine screening of the intronless gene Kir6.2.

In the course of a research program on genetic determinants of obesity, the search for mutations in the human mitochondrial uncoupling protein (UCP) gene was initiated. The UCP gene was investigated on the basis of an association between a high weight gain in obese patients and the 8.3-kb allele of a Bc/I polymorphism 3 kb upstream of the gene (9). The method described in this report was applied to screen the promoter sequence of the human mitochondrial UCP gene (1) for the presence of new mutations in a population of 227 massively obese patients (body mass index [BMI] > 40 kg/m²) and 99 nonobese subjects (BMI < 27 kg/m²).

For all the different mutations studied, genomic DNA was amplified on a PTC-200™ Thermal Cycler (MJ Research, Watertown, MA, USA) using each DNA fragment’s specific oligonucleotides extended by the universal -21 M13 (TGTTAAACGACGAGTCT) and M13 Reverse (CAGGAAACAGCCTCCGGTTG) sequences at their 5’ ends. Amplifications were carried out in a total volume of 50 μL with 100 ng of genomic DNA, 1 U of Taq DNA Polymerase (Perkin-Elmer, Norwalk, CT, USA) and 0.25 μM of each primer. PCR cycling conditions for the GCK, Kir6.2 and SUR genes have been previously described (7–9). Primers M13 Reverse UCP2 (5’ CAGGAAACAGCCTCCGGTTG) and -21 M13 UCP5 (5’ TGTAAACGACGAGTCT) previously reported through radioactive and/or silver-staining SSCP methods (Table 1). For the GCK gene, 5 mutations in Exons 3, 7 and 9 were detected in families with MODY, where these mutations were originally described. In other patients with late-onset diabetes of the young (MODY) (3). This gene was chosen for two reasons. The first is that the availability of a large number of different mutations (over 100 have been reported all over the world). Second, routine screening of the intronless gene Kir6.2.

In the course of a research program on genetic determinants of obesity, the search for mutations in the human mitochondrial uncoupling protein (UCP) gene was initiated. The UCP gene was investigated on the basis of an association between a high weight gain in obese patients and the 8.3-kb allele of a Bc/I polymorphism 3 kb upstream of the gene (9). The method described in this report was applied to screen the promoter sequence of the human mitochondrial UCP gene (1) for the presence of new mutations in a population of 227 massively obese patients (body mass index [BMI] > 40 kg/m²) and 99 nonobese subjects (BMI < 27 kg/m²).

For all the different mutations studied, genomic DNA was amplified on a PTC-200™ Thermal Cycler (MJ Research, Watertown, MA, USA) using each DNA fragment’s specific oligonucleotides extended by the universal -21 M13 (TGTTAAACGACGAGTCT) and M13 Reverse (CAGGAAACAGCCTCCGGTTG) sequences at their 5’ ends. Amplifications were carried out in a total volume of 50 μL with 100 ng of genomic DNA, 1 U of Taq DNA Polymerase (Perkin-Elmer, Norwalk, CT, USA) and 0.25 μM of each primer. PCR cycling conditions for the GCK, Kir6.2 and SUR genes have been previously described (7–9). Primers M13 Reverse UCP2 (5’ CAGGAAACAGCCTCCGGTTG) and -21 M13 UCP5 (5’ TGTAAACGACGAGTCT) previously reported through radioactive and/or silver-staining SSCP methods (Table 1). For the GCK gene, 5 mutations in Exons 3, 7 and 9 were detected in families with MODY, where these mutations were originally described. In other patients with late-onset diabetes of the young (MODY) (3). This gene was chosen for two reasons. The first is that the availability of a large number of different mutations (over 100 have been reported all over the world). Second, routine screening of the intronless gene Kir6.2.
set NIDDM, we detected 3 and 2 previously described mutations in the human SUR and Kir6.2 genes, respectively. In Figure 1, three patterns of SSCP variants were shown. For the GCK (Exon 7, GGG→AGG), SUR (Exon 22) and Kir6.2 (Leu270Val) genes, the mutations were detected on the sense strand (blue peaks for patient 2599), the antisense strand (green peaks for patient 2) and both strands (blue and green peaks for patient 1), respectively. The percentages in which mutations were detected on the sense, antisense or both strands are given in Table 1. All mutations screened for were readily detected using our SSCP protocol. Moreover, a C to T mutation in Exon 3 of the GCK gene, so far undetected by radioactive SSCP in earlier reports, was unambiguously detected here using our protocol. For Exon 9 of the GCK gene, a mutation in a 403-bp fragment was detected using silver-staining SSCP, only after size reduction by digestion of the amplified product with the restriction endonuclease SphI. Using the fluorescence-based SSCP method described in this report, the enzymatic digestion was not necessary for reproducible detection of this mutation.

After evaluation of this method by screening for known mutations, the protocol was applied to screen for new mutations in the promoter sequence of the UCP gene. A single heterozygous point mutation in a 330-bp PCR fragment was detected with this method in patient 4, as shown in Figure 2a. An additional peak appears on the electropherogram. Sample 3 is a negative control. The DNA variation was then identified by direct sequencing of the PCR products on the DNA sequencer using a standard dye primer protocol (Perkin-Elmer). The mutation was shown to be a C to T transition at position -305 of the promoter sequence (Figure 2b). This transition abolishes an HphI restriction site, therefore creating a new HphI restriction fragment-length polymorphism and was found with a frequency of 8.59% in the obese patients and of 8.58% in nonobese subjects (P ≥0.05, not significant).

Unlike end-labeled primers, PCR products were also labeled by direct incorporation of fluorescent TAMRA dUTP during amplification. Although all three mutations in Exon 7 of the GCK gene were detected, generally this labeling procedure did not give reproducible results (absence of mutation peaks). This may be caused by mobility shifts of single strands in the gel because of different incorporation rates of the TAMRA dUTP dye. In addition, we failed to detect the UCP promoter mutation using the TAMRA dUTP internal labeling upon SSCP screening. This observation is in contrast to recently published data by Iwahana et al., who reported that the internal labeling is a robust method to use with SSCP methodology (6). This discrepancy might be due to the variable sensitivity of different DNA fragments (single strands) to unequal TAMRA dUTP incorporation.

In conclusion, we report the development of a quick and sensitive SSCP protocol based on a fluorescence end-labeling procedure using universal M13 primers, with the use of an automated DNA sequencer for detection. The advantages of our use of primers extended by universal M13 sequences (-21 M13 and M13 Reverse) are: (i) the same couple of fluorescently labeled...
primators may be used to detect all DNA fragments, thus reducing the cost of this method considerably; (ii) the double labeling allows the distinction of the two single strands of a DNA fragment on the DNA sequencing machine; (iii) the PCR fragments can be readily sequenced using the M13 dye primers without further modifications; and (iv) this protocol can generally be used with any automated, fluorescence-based sequencer, allowing a widespread application in the gene mutations search.

Moreover, we found that at least for the fragments tested in this study, the same electrophoresis conditions, concentration of gel matrix and temperature could be applied for fragments of 200–400 bp. For smaller DNA fragments (100–200 bp), a denser gel matrix (0.5x or 0.8x MDE) may be used. Using these experimental conditions, every tested mutation was reproducibly detected without the necessity for intensive re-optimization for each DNA fragment. Therefore, this method may be especially interesting in cases where genes with many exons have to be screened.

REFERENCES


DNA Sequencing with Modular Primers Using a Two-Step Protocol with Thermostable Polymerase at the Second Step

The recent development of modular primers for DNA sequencing is intended to eliminate the primer-synthesis step, the main bottleneck in primer walking (3,5). Modular primers can be assembled from three 5-, 6- or 7-mer modules selected from a pre-synthesized library of as few as 1000 oligonucleotides. Apart from saving time and cost (of both primers and labor), closed-loop automation would also eliminate the need for human intervention between the walks. The modular primer technique was originally developed with Sequenase® (Amersham, Arlington Heights, IL, USA) (1–3,5), which performed better than other polymerases when used with modular primers where the front module was 5- or 6-mer. Thermostable polymerases like Taq, SequiTherm™ (Epiconce Technology, Madison, WI, USA) and Thermo Sequenase™ (Amersham) can

Address correspondence to Philippe Boutin, CNRS EP10, Genetics of Multifactorial Diseases, Institut Pasteur de Lille, 1 rue du professeur Calmette BP 245, 59045 Lille Cedex, France. Internet: p.boutin@xenope.univ-lille2.fr

Received 2 December 1996; accepted 10 March 1997.


CNRS EP 10
Institut Pasteur de Lille and Faculté de Médecine
C.H.U. de Lille
Lille, France

BioTechniques 23:362-368 (September 1997)