Multiplex SSCP and Heteroduplex Analysis with Southern Hybridization for Large-Scale Mutation Detection

R. Pogue, S. West, and K. Bushby

Department of Human Molecular Genetics, University of Newcastle-upon-Tyne, Ridley Building, Claremont Road, Newcastle-upon-Tyne, NE1 7RU, United Kingdom

Received April 20, 1998; accepted August 5, 1998

We have developed a modification of the single-strand conformational analysis and heteroduplex analysis methods of mutation detection, with the intention of applying them to genetic diseases involving large genes or multiple genes producing a similar phenotype. The technique involves electrophoresing up to 10 or more DNA fragments on a polyacrylamide gel, followed by bidirectional Southern blotting and individual examination by hybridization. This can reduce the time involved in mutation detection by more than 50%. We confirmed the validity of our approach by detecting 90% of mutations in a blind study of previously characterized mutations in the adenomatous polyposis coli (APC) gene that underlies familial adenomatous polyposis.

INTRODUCTION

Defining the mutation has become the ultimate diagnostic test in a range of genetic disorders. For genetically homogeneous disorders with single or common mutations, mutation detection can be easily translated into a straightforward diagnostic test. However, searching for unknown mutations can be an onerous task, especially in disorders where the phenotype can be caused by a range of mutations in a large gene (Franz et al., 1995; Rodenhiser et al., 1997) or where there is locus heterogeneity. We are performing mutation detection on samples from patients with recessive limb-girdle muscular dystrophy (LGMD), of which there are currently eight subtypes, with five of the genes characterized (Bushby, 1996; Anderson, 1996). These genes have 55 exons among them.

Most mutation detection techniques in common usage represent a compromise between sensitivity and time efficiency (Prosser, 1993; Grompe, 1993). Single-strand conformational polymorphism analysis (SSCP) (Orita et al., 1989a, b; Glavac and Dean, 1993) and heteroduplex analysis (HA) (Glavac and Dean, 1995) are mutation detection techniques that rely on detecting changes in the physical properties of DNA caused by the presence of sequence changes.

Both these techniques have the advantages of being rapid, cheap, and easy to perform. However, it is accepted that they can offer no better than approximately 70–80% (or lower for HA) mutation detection efficiency under a single set of conditions and that at least two sets of electrophoretic conditions are required for 100% detection (Cotton, 1997). Optimum fragment length for examination is up to 250 bp, and efficiency decreases above this size.

Our method of choice in the study of LGMD has been a combination of SSCP, and HA. These have been
performed nonradioactively on MDE polyacrylamide gels, with examination by silver staining (Fig. 1) (Dockhorn-Dworniczak et al., 1991).

These techniques, performed individually exon by exon, are extremely time-consuming, so we have devised a modified method to decrease the time involved. We decided to multiplex the analyses on a large scale. We have developed a method of blotting the DNA from the SSCP/HA gels and then performing Southern hybridizations to examine each exon separately. In the work reported here, we describe the validation of the technique through the study of a group of 20 patients with known mutations in the APC gene that underlies familial adenomatous polyposis coli (FAP) (Groden et al., 1991). The object of the exercise was to determine the percentage of mutations that could be detected by this technique and to show that no decrease in the resolution of SSCP and HA is suffered when compared with our original method.

**MATERIALS AND METHODS**

The DNA samples used were from 20 individuals carrying previously characterized pathological mutations in the APC gene that causes FAP, an autosomal dominantly inherited predisposition to colon cancer. The mutations were dispersed over 10 exons of the gene, and these exons ranged in size from 84 to 434 nucleotides (Groden et al., 1991). All patients had mutations that had been previously identified, by heteroduplex analysis, by the protein truncation test (PTT) (Roest et al., 1993), or by SSCP analysis. The exact mutation previously found in each particular sample was not known to the laboratory investigator (R.P.) until the analysis was complete.

All 10 exons were amplified for each patient using PCR (100–200 ng genomic template, 30 μl reaction). The 10 PCR products for each patient were then pooled. Sixty microliters of the pooled products were then taken and added to an equal volume of SSCP loading buffer (95% formamide, 20 mM EDTA, 20 mM NaOH, 0.5% xylene cyanol, 0.5% bromophenol blue). This was denatured by heating at 95°C for 5–7 min and then plunged in ice (in our experience, some renaturation of the strands does occur, thus providing the duplexes required for HA). Eighty microliters of this was loaded on a mutation detection enhancement (MDE) gel (Flowgen, 16 x 20 cm, containing 5% glycerol). Electrophoresis was performed in 0.6 x TBE at 140 V for 1 h followed by 300 V overnight at 10°C. These have previously been reported as the single set of conditions with the highest mutation detection efficiency for SSCP (C. Beljourd, Paris, pers. comm., June 1997).

When electrophoresis was completed, bidirectional Southern blotting was performed overnight using 2 x SSC buffer. Bidirectional blotting involved placing a piece of Hybond-N + membrane (Amersham) above and below the gel, so that the DNA was drawn in two directions at once, providing twice as many filters, thus halving the time involved in hybridization and probing. After being blotted overnight, the membranes were then placed in 0.4 M NaOH for 10 min to denature the double-stranded DNA, followed by equilibration for 10 min in 2 x SSC.

The blots were probed overnight using a random-labeled aliquot of each of the original PCR products for each exon in turn. They were then washed down to 200 counts per second or less and exposed to photographic film (Kodak Biomax, –80°C overnight).

**RESULTS**

Of the 20 mutations known to be present in our patient samples, 18 were shown as SSCP and/or heteroduplex changes. This represents a 90% rate for detection of mutations using a combination of the two techniques. The two mutations that were not found were also not seen on a normal silver-stained SSCP gel using the same set of running conditions, indicating that the inability to show changes for these mutations was not due to any loss in sensitivity caused by our modifications. Originally, one of these mutations had been found by PTT analysis and the other by HA. Both

### TABLE 1

**DNA Fragments and Mutations Used in This Study**

| APC exon fragment | Size (basepairs) | Nucleotide change | Original detection method | Detected by this method? |
|------------------|-----------------|------------------|--------------------------|--------------------------|
| 3                | 202             | AAGA del         | HA                       | Y                        |
| 6                | 84              | AG ins           | HA                       | Y                        |
| 8                | 99              | G->A             | SSCP                     | Y                        |
| 9                | 379             | CT del           | HA                       | Y                        |
| 14               | 215             | A ins            | HA                       | Y                        |
| 14               | 215             | C del            | HA                       | Y                        |
| 14               | 215             | G del            | HA                       | Y                        |
| 14               | 215             | A del            | HA                       | Y                        |
| 14               | 215             | C->T             | SSCP                     | Y                        |
| 15D              | 382             | T->G             | PTT                      | N                        |
| 15E              | 429             | AAGAA del        | HA                       | Y                        |
| 15E              | 429             | TCAA del         | HA                       | Y                        |
| 15E              | 429             | ACAAA del        | HA                       | Y                        |
| 15F              | 434             | AA del           | HA                       | Y                        |
| 15G              | 386             | AAAGA del        | HA                       | Y                        |
| 15G              | 386             | C->T             | HA                       | N                        |
| 15G              | 386             | AAAA del         | PTT                      | Y                        |
| 15H              | 420             | TG del           | HA                       | Y                        |
| 15H              | 420             | AG del           | PTT                      | Y                        |

Note: Information taken from Groden et al. (1991).
were missense mutations. The fragments in which these changes were situated are 382 and 386 bp. At least one other mutation was successfully identified in each of these fragments. Table 1 lists the nature of each of the mutations used in the study and the size of the DNA fragments in which they are situated.

**DISCUSSION**

We have devised a multiplex Southern blot SSCP/HA to reduce the workload involved in performing mutation analysis in large regions of DNA and have validated it in this sample of patients with APC mutations (Fig. 2). The APC gene has 16 exons, one of which is 6.5 kb long and is normally analyzed by SSCP/HA in 23 overlapping PCR products. This method should also be of particular use to those examining genetically heterogeneous conditions. We did not use silver staining in the visualization of the results, as the mobility of DNA on an SSCP gel cannot be predicted and the resolution of double-stranded DNA would also have been lost.

Our technique has been shown to cause no loss in sensitivity in comparison with standard SSCP/HA. Ninety percent of mutations present in these sam-
samples were detected in this study. Some of the DNA fragments in which mutations were identified are considerably larger than the recommended size for SSCP and heteroduplex analyses, indicating that our conditions are very sensitive and reliable for routine mutation detection in disease genes. We predict that the two mutations not seen as band-shifts would be picked up under different electrophoretic conditions. The performance of SSCP/HA is known to be partly related to base composition (Ravnik-Glavac et al., 1994), and it has been reported that mutations close to primers can be particularly difficult to detect (T. Hamzehloei, Ph.D. thesis, 1996). Although each exon is still examined separately, a considerable amount of time is saved by being able to reprobe the blots each day. Since the DNA transfer occurs passively, the time saving is further increased by performing bidirectional blots. Though it was not done here, one could also increase the number of fragments on the gel by multiplexing two or more fragments in each PCR tube. We believe that having optimized the technique, the hands-on time involved in SSCP/HA is reduced by at least 50%. Furthermore, the cost is reduced since much less acrylamide (MDE) than normal is used—in this case 90% less. We believe that the only limitation on the number of DNA fragments that can be examined by this method is the capacity of the wells in the gel.

The method has disadvantages, such as the use of radioisotopes, whereas the current trend in mutation detection techniques is to remove the requirement for radioactivity (Cotton, 1997). However, there may exist the possibility for nonradioactive probing, such as the use of digoxigenin.

We believe that the technique described here will become a valuable tool in the analysis of large genes or genes with multiple different mutations underlying genetically heterogeneous disorders, because of the high throughput without loss of sensitivity. We are currently using multiplex Southern blot SSCP routinely in our continued study of limb-girdle muscular dystrophy.

ACKNOWLEDGMENTS

The authors thank Mr. Andy Curtis and Dr. Rumaisa Bashir for technical advice and Mr. Simon Hill for his contribution to optimizing the technique. This work is funded by the Muscular Dystrophy Group of Great Britain and Northern Ireland.

REFERENCES

Anderson, L. V. B. (1996). Optimized protein diagnosis in the autosomal recessive limb-girdle muscular dystrophies. Neuromusc. Disorders 6(6): 443–446.

Bushby, K. M. D. (1996). Towards the classification of the autosomal recessive limb-girdle muscular dystrophies. Neuromusc. Disorders 6(6): 439–441.

Cotton, R. G. H. (Ed.) (1997). "Mutation Detection," Oxford Univ. Press, Oxford.

Dianzini, I., Camaschella, C., et al. (1993). Dilemmas and progress in mutation detection. Trends Genet. 9(12): 403–405.

Dockhorn-Dworniczak, B., et al. (1991). Non-isotopic detection of single strand conformational polymorphism (PCR-SSCP): A rapid and sensitive technique in diagnosis of phenylketonuria. Nucleic Acids Res. 19(9): 2500.

Franz, W. M., Frey, N., Muller, O., Kubler, W., and Katus, H. A. (1995). A transgenic animal model: New possibilities for cardiovascular research. Z. Kardiol. 84 (Suppl. 4): 17–32. [In German]

Glavac, D., and Dean, M. (1993). Optimisation of the single-strand conformation polymorphism (SSCP) technique for detection of point mutations. Hum. Mutat. 2: 404–414.

Glavac, D., and Dean, M. (1995). Applications of heteroduplex analysis for mutation detection in disease genes. Hum. Mutat. 6: 281–287.

Groden, J., Thilveris, A., et al. (1991). Identification and characterisation of the familial adenomatous polyposis coli gene. Cell 66: 589–600.

Grompe, M. (1993). The rapid detection of unknown mutations in nucleic acids. Nat. Genet. 5: 111–117.

Orita, M., Iwahana, H., Kanazawa, H., and Sekiya, T. (1989a). Detection of polymorphism of human DNA by gel electrophoresis as single strand conformation polymorphism. Proc. Natl. Acad. Sci. USA 86: 2766–2770.

Orita, M., Suzuki, Y., Sekiya, T., and Hayashi, K. (1989b). A rapid and sensitive detection of point mutations and genomic polymorphism using polymerase chain reaction. Genomics 8: 271–278.

Prosser, J. (1993). Detecting single-base mutations. TibTech 11: 238–246.

Ravnik-Glavac, M., Glavac, D., and Dean, M. (1994). Sensitivity of single-strand conformation polymorphism and heteroduplex method for mutation detection in the cystic fibrosis gene. Hum. Mol. Genet. 3(5): 801–807.

Rodenhiser, D. I., Andrews, J. D., Mancini, D. N., Jung, J. H., and Singh, S. M. (1997). Homonucleotide tracts, short repeats and CpG/CpNpG motifs are frequent sites for heterogeneous mutations in the neurofibromatosis type 1 (NF1) tumour-suppressor gene. Mutat. Res. 373(2): 185–195.

Roest, P., Roberts, R., van der Luijt, A., Heikoop, J., van Ommen, G., and Dunnen, J. J. (1993). Protein truncation test to rapidly screen the DMD gene for translation terminating mutations. Neuromusc. Disorders 3: 391–394.

White, M. B., et al. (1992). Detecting single base substitutions as heteroduplex polymorphisms. Genomics 12: 301–306.