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'Broad-range' DGGE for single-step mutation scanning of entire genes: application to human phenylalanine hydroxylase gene

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Attempts to isolate human genes and characterize molecular defects underlying inherited diseases are in rapid progress. The evolving possibilities of improved diagnosis by analysis at the DNA level have prompted the need for efficient methods that enable rapid detection of mutations. For diseases caused predominantly by large gene deletions or expansion of a trinucleotide repeat, simple PCR-based assays are now available for diagnosis and evaluation of carrier status. However, for diseases that are caused primarily by point mutations, e.g. cystic fibrosis, β-thalassaemia, and phenylketonuria (PKU), no mutation scanning method has yet proven satisfactory for rapid and comprehensive gene analysis in single individuals, and DNA sequencing still seems impracticable for determining whether a particular gene in an individual harbours a point mutation or not. For these reasons, diagnostic approaches have to a wide extent relied on methods for recognition of previously identified mutations (for a recent review see reference 1). Owing to a large number of different mutations and a marked divergence in mutational spectra between different populations, the sensitivity of these methods is often low. In this report we describe a modification of denaturing gradient gel electrophoresis (DGGE) (2), which we term 'broad-range' DGGE, that allows simultaneous analysis of multiple PCR-amplified DNA fragments, enabling rapid one-step scanning of entire genes for the presence or absence of any point mutation.

The mutation-resolving power of DGGE relics on a physical separation between similar DNA fragments differing in melting properties due to differences in nucleotide composition (2). Two modifications greatly increase the sensitivity of the technique: Attachment of a thermostable GC-clamp to one of the ends of the DNA fragment (3), and analysis of heteroduplex molecules, i.e. hybrids formed between mutant and prototype strands (4). The clear advantages of DGGE for population studies have been substantiated by a number of recent studies, demonstrating a mutation detection efficiency near 100%. However, when there is a need to test a particular gene in only one individual, the conventional methodology is not rational due to the fact that different parts of the gene are generally analysed under different experimental conditions to optimize resolution of mutations. We have attempted to overcome this limitation by exploiting the flexibility of DGGE with respect to two critical parameters: gradient range and running time.

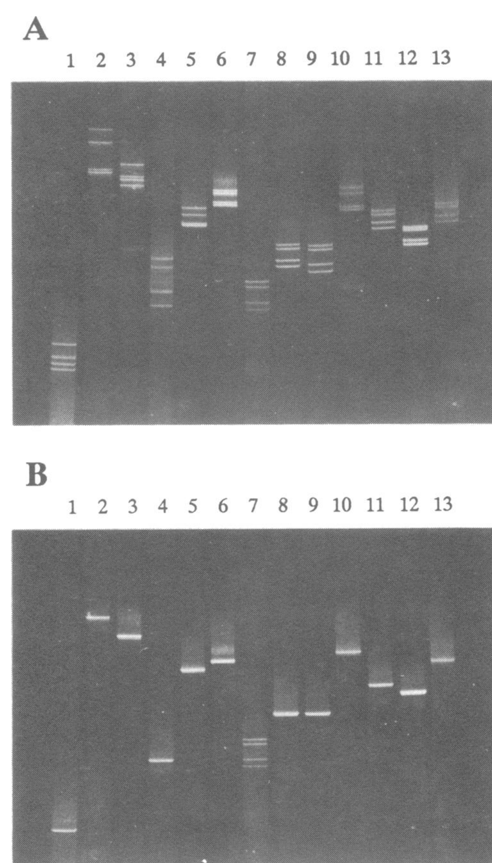

Figure 1. Simultaneous DGGE analysis of the entire coding sequence and splice junctions of the human PAH gene. The 13 exonic fragments were amplified from genomic DNA in individual tubes under the same PCR conditions (40 rounds of thermal cycling at 94°C for 60 s, 55°C for 90 s, and 72°C for 60 s). Heteroduplexes were generated by boiling for 5 min, and subsequent reannealing in two 1 h steps at 65°C and 37°C, respectively. 15 μl of each sample was subjected to electrophoresis in a 0–80% DGG for 4.5 h at 150 V and 60°C. Primer sequences were as previously described (7). (**A**) Exons were amplified from individuals heterozygous for a known mutation in the relevant region of the gene. (**B**) One-step carrier analysis by mutation scanning of the entire PAH gene in an individual, demonstrating a mutation in exon 7.

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Our panel of DNA fragments included 19 GC-clamped PCR products (183 to 396 bp in length) containing one or two relevant melting domains with melting temperatures of 65°C to 78°C, as predicted by the computer algorithm MELT87 (5). 59 known point mutations in these fragments (at least two mutations in each fragment) were analysed as heteroduplexes in a 6% polyacrylamide gel containing a 'broad-range' (0°80%) gradient of denaturant. Electrophoresis was performed at 150 V with running time varying from 1 to 8 h in 1 h increments. Our results showed that once a fragment has reached the concentration of denaturant at which it undergoes an abrupt reduction in mobility, the electrophoretic band pattern becomes almost time independent. This allows prolonged electrophoresis without loss of the resolution of mutations. All 59 point mutations in our panel were clearly revealed by the presence of more than one band in the gel after 5 h of electrophoresis at 150 V. We conclude that multiple DNA fragments can be scanned for mutations by heteroduplex analysis in a 0−80% DGG where the running time is determined as the time it takes for the slowest-migrating fragment to reach its final position in the gel.

We have adapted this strategy to mutation scanning of the human phenylalanine hydroxylase (PAH) gene. More than 125 mutations in this gene have been characterized causing PKU and related hyperphenylalaninemias. Recently, we have established the mutational spectra associated with PAH deficiency in Denmark and Sicily (6, 7). An overall mutation detection efficiency of 98.8% was achieved by using a conventional DGGE assay. Genomic sequences covering the entire coding sequence and splice junctions of the PAH gene were amplified and GC-clamped, resulting in 13 PCR products (199 to 369 bp in length) with relevant domains showing predicted melting temperatures between 67°C and 76°C. By using one general DGGE protocol (0−80% DGG, 4.5 h at 150 V and 60°C) we have tested 75 different point mutations distributed over the entire PAH gene. All mutations were clearly resolved, enabling simultaneous analysis of all 13 PAH-coding fragments (Figure 1A). The resulting procedure is a simple non-radioactive assay that allows us to scan the PAH gene for, in theory, any point mutation in any individual within a single day. We use this approach for rapid genotyping of newborns with hyperphenylalaninemia and potential carriers in families at risk (Figure 1B).

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