

Improved mutation detection in GC-rich DNA fragments by combined DGGE and CDGE

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ABSTRACT

Denaturing gradient gel electrophoresis (DGGE) has proven to be a powerful pre-screening method for the detection of DNA variants. If such variants occur, however, in DNA fragments that are very rich in G and C, they may escape detection. To overcome this limitation, we tested a novel gel system which combines DGGE and constant denaturant gel electrophoresis (CDGE), as it might have the advantages of both methods. Indeed, this combination had the advantages of both methods, good separation of heteroduplex molecules and prevention of total strand dissociation, and it proved successful in the detection of DNA variants in several GC-rich fragments.

INTRODUCTION

Denaturing gradient gel electrophoresis (DGGE) of GC-clamped DNA fragments (1–5) is a powerful tool to detect small mutations in PCR-amplified DNA fragments. When correctly designed and applied the mutation detection rate is virtually 100% (4–6). Application of DGGE to GC-rich fragments, however, has proved very difficult. Variants are hard to detect, presumably because of the small temperature difference between the melting temperature (T_m) of the GC-rich DNA fragment and that of the clamp. Therefore, the part of the denaturing gradient in which the mutation will become visible is very small and, because of the high melting temperature, will be at the very bottom of the gel in which the fragment melts completely, implying the risk that the fragment will run off the gel. This makes reliable mutation detection of GC-rich fragments extremely difficult. In some cases, complete strand dissociation can be prevented by adjusting the length of the GC clamp, thus reducing the mobility of the partially melted migrating fragment (7). For extremely GC-rich sequences (T_m > 80°C), however, a longer clamp is still not sufficient to prevent total strand dissociation. Several solutions have been suggested to this problem. One might be the use of primers with a chemical clamp instead of a GC clamp. The chemical clamp should prevent complete strand dissociation of the fragments, which is the main problem in mutation detection by DGGE in GC-rich fragments. Such primers have a photoactivatable intercalating agent (psoralen) at their 5'-end which stabilises the PCR fragment by covalently binding the two strands (8,9). We tested the use of such a chemical clamp, however, several mutations remained undetectable in GC-rich fragments. Another alternative might be to use constant denaturant gel electrophoresis (CDGE) instead of DGGE. In CDGE, a single denaturing condition is used, which is between the T_m of the fragment and that of the GC clamp (10–12). CDGE, however, is not the method of choice for searching for unknown mutations in relatively large DNA fragments with multiple melting domains, since each variant/domain requires a specific electrophoretic condition for optimal resolution (13). Furthermore, heteroduplex molecules, which melt at a lower temperature than homoduplexes, may not be detected, since all fragments may melt immediately in the relatively high concentration of denaturant. If heteroduplex molecules are not detected, the mutation detection rate decreases dramatically. Another alternative has been reported by Guldberg and colleagues (14), who treated template DNA with sodium bisulphite to lower the T_m of GC-rich DNA fragments. The disadvantage of this method is that for the modified strand extra primers must be designed. Also, methylation of DNA may give false positive results.

To facilitate detection of mutations in GC-rich DNA fragments, we compared DGGE with CDGE and tested the use of a combination of DGGE and CDGE and compared this with the use of the individual methods. The combined DGGE/CDGE system was chosen as it might have the advantages of both systems; heteroduplex molecules may become visible in the DGGE component, while the CDGE component should prevent complete strand dissociation.

MATERIALS AND METHODS

DNA amplification

PCR amplification of *RET* exons 1 and 4 and a part of *MSH6* exon 1 was carried out as previously described (15,16). A 60 bp GC clamp was attached to one of the primers. The primer sequences are available on request.

Denaturing gradient gel electrophoresis (DGGE)

An aliquot of 20 µl of the amplified product was transferred to a 0.75 mm thick 9% polyacrylamide (PAA) gel (acrylamide:bisacrylamide 37.5:1) containing a 50–85% denaturing gradient of urea–formamide [100% urea–formamide (UF) contains 7 M urea and 40% deionized formamide]. DGGE was

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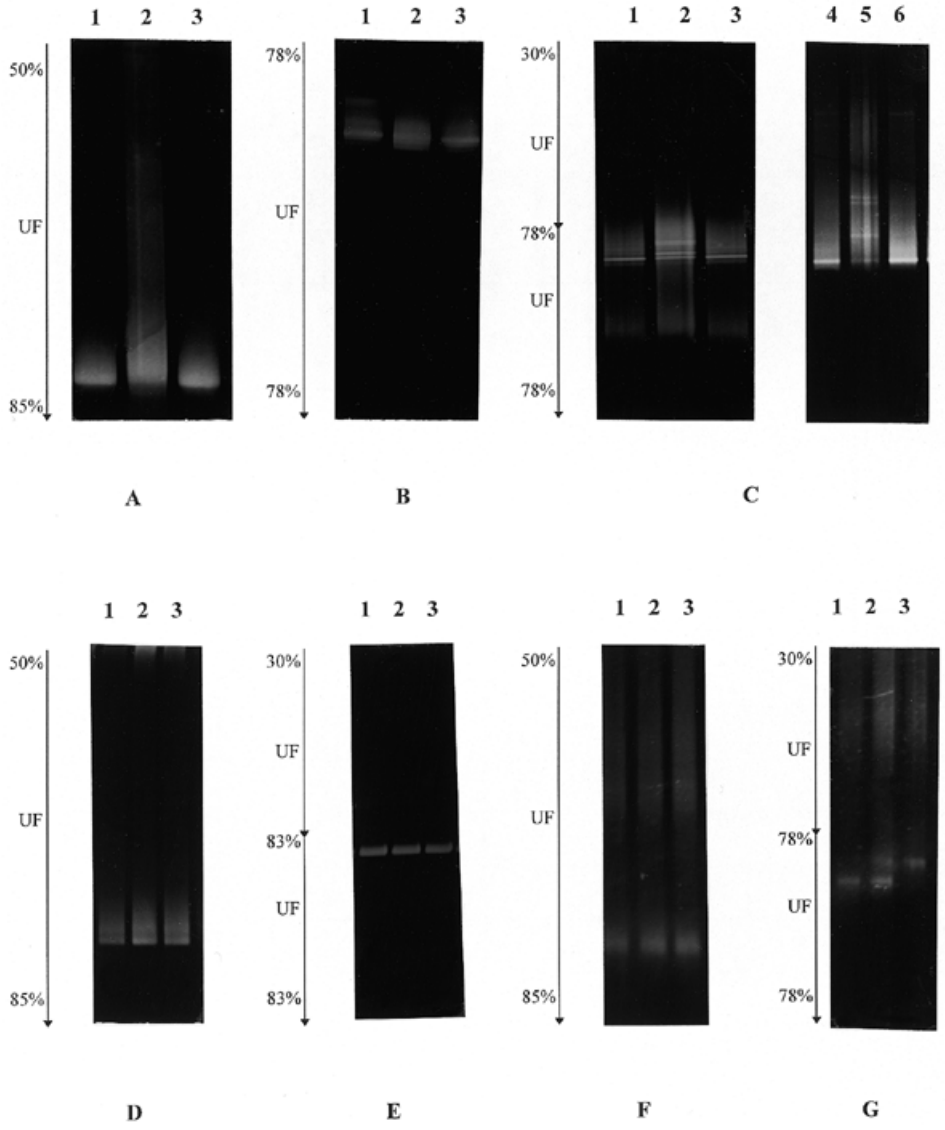

Figure 1. Mutation analysis of GC-rich fragments of the *RET* and *MSH6* genes. (**A**) DGGE and (**B**) CDGE analysis of *RET* exon 4 and its flanking intronic sequence from two control individuals (lanes 1 and 3) and from a Hirschsprung patient who was a mutation carrier (Glu235Lys) (lane 2). The mutation could not be convincingly demonstrated in either gel. (**C**) Combined denaturing gel electrophoresis of *RET* exon 4 on the same sample set (lanes 1–3) and on DNA samples from some additional Hirschsprung patients (lanes 4–6). The mutated PCR products (lanes 2 and 5) give the expected four bands. (**D**) DGGE analysis of *RET* exon 1 and its flanking intronic sequence in three individuals. All bands are fuzzy and diffuse in the DGGE gel. (**E**) Combined denaturing gel electrophoresis of *RET* exon 1 from the same three individuals shows sharp and focused bands. (**F**) DGGE analysis of a part of *MSH6* exon 1 in three individuals. All bands are fuzzy and a neutral variant could not be demonstrated with regular DGGE (lanes 2 and 3). (**G**) Combined denaturing gel electrophoresis on the same samples. A clear difference between the homoduplex bands of the variant PCR products (lanes 2 and 3) and that of the normal product (lane 1) can be seen.

performed in 0.5× TAE (1× TAE = 40 mM Tris-acetate pH 8.0, 20 mM Na-acetate, 1 mM Na_2EDTA) at 60°C for 1500 V/h using a 19 cm gel. The separation pattern was visualised by ethidium bromide (EtBr) staining for 10 min and UV trans-illumination of the gel.

Constant denaturant gel electrophoresis (CDGE)

An aliquot of 20 µl of the amplified product was transferred to a 9% PAA gel containing 78% UF. CDGE was performed in 0.5× TAE at 60°C for 1500 V/h using a 19 cm gel. The gel was stained with EtBr.

Combined denaturing gel electrophoresis

An aliquot of 20 µl of the amplified product was transferred to a 9% PAA gel containing a combined denaturing gradient. For *RET* exon 4 and the part of *MSH6* exon 1, the gel consisted of a CDGE part (78% UF) in the lower 10 cm of the gel and a DGGE part (30–78% UF) in the upper 9 cm of the gel. Electrophoresis was performed in 0.5× TAE at 60°C for 1500 V/h. For *RET* exon 1, the gel consisted of a CDGE part (83% UF) in the lower 10 cm of the gel and a DGGE part (30–83% UF) in the upper 9 cm of the gel. Electrophoresis was performed in 0.5× TAE at 58°C for 1500 V/h. The gel was stained with EtBr.

RESULTS AND DISCUSSION

To test whether the detection of mutations by DGGE and CDGE can be improved by a combined DGGE/CDGE gel system, we used exon 4 of the *RET* gene, which has a very high GC content (71%), giving a T_m value of 84°C, and in which a mutation (Glu235Lys) was previously found because of an abnormal DGGE pattern (Fig. 1A, lane 3).

Figure 1A shows the DGGE analysis of *RET* exon 4 from two controls (lanes 1 and 3) and from the mutation carrier (lane 2). A 60 bp GC clamp was used, since attachment of a 40 bp GC clamp to the fragment did not lead to a focused band(s) in the gel. Even when the fragments were melted, they kept on running through the gel (data not shown). With longer electrophoresis times bands run off the gel. Although the DGGE patterns of the normal (lanes 1 and 3) and mutated (lane 2) PCR products show some differences, the mutation could not be convincingly demonstrated.

Figure 1B shows the CDGE analysis of *RET* exon 4 on the same test panel. As mentioned, detection of heteroduplex molecules by CDGE may be a problem. There is a difference between the mutated PCR product (lane 2) and the control products (lanes 1 and 3), but again the mutation could not be convincingly demonstrated.

Figure 1C shows the results of the combined DGGE and CDGE gel system for *RET* exon 4. The combined gel system improved detection of the mutation remarkably. Using the same DNA samples as used for DGGE and CDGE, one focused band in the lanes containing the normal PCR fragments can be seen (lanes 1 and 3), whereas the mutated PCR products (lane 2) give the expected four bands (two heteroduplexes and two homoduplexes). When we screened a large number of Hirschsprung patients, another variant in *RET* exon 4 was readily detected (Fig. 1C, lane 5).

To further demonstrate the use of this combined gel system, we applied it to *RET* exon 1 (77% GC, T_m 85°C) and to part of *MSH6* exon 1 (74% GC, T_m 84°C). Figure 1D and E shows a comparison of DGGE analysis with the combined gel system in *RET* exon 1. The combined gel system remarkably improved the band resolution of this fragment, showing much sharper bands in the combined gel (Fig. 1E) than in the corresponding DGGE gel (Fig. 1D). No variant has yet been detected in this fragment. Figure 1F and G shows the same comparison of analysis of the part of *MSH6* exon 1. Screening this fragment with the combined DGGE/CDGE gel system we found a neutral variant. Lane 2 shows a heterozygous individual, whereas lane 3 shows a homozygous individual. Although in both DGGE and combined DGGE/CDGE fuzzy bands are observed, the resolution of the combined gel system proved to be much better. A clear difference between the homoduplex bands of the variation (lanes 2 and 3) and that of the normal product (lane 1) can be seen (Fig. 1G). No heteroduplex bands were detected.

Based on these results and on additional analysis of several other GC-rich fragments, such as *GDNF* exon 1 and *BRCA2* exon 1 (data not shown), we conclude that a combined DGGE and CDGE gel system is an effective prescreening method for mutations in GC-rich DNA fragments. Which constant denaturant concentration should be used can be determined either from the theoretical melting curve or preferably from a perpendicular DGGE experiment.

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