Minisatellite isoalleles can be distinguished by single-stranded conformational polymorphism analysis in agarose gels

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ABSTRACT

Minisatellite isoallelism, i.e. the occurrence of minisatellite alleles with different internal sequence composition but indistinguishable length, is a common limitation of minisatellite allele length analysis. Internal sequence variation can be used to distinguish such isoalleles, provided that detailed sequence knowledge of its basis is available. We now show that minisatellite isoalleles can also be simply resolved by single-stranded conformational polymorphisms (SSCP) arising during agarose gel electrophoresis. SSCP on agarose gels can be used to distinguish minisatellite isoalleles either after PCR amplification, or by standard Southern blot analysis of genomic DNA.

Hypervariable minisatellites are used as highly informative markers in linkage mapping and individual identification through Southern blot length analysis. However, agarose gel electrophoresis cannot distinguish similar sized minisatellite alleles or alleles of the same length but different internal sequence; individuals containing such isoalleles can thus be mis-scored as homozygous (1, 2). Minisatellite internal sequence variation can be assayed by sequencing (e.g. 3), restriction mapping (e.g. 4) and minisatellite variant repeat mapping by PCR (MVR-PCR) (2). Unfortunately, many of the usual approaches used for identifying sequence variation are not suitable for the analysis of large minisatellite alleles. MVR mapping, although simple to perform, requires detailed sequence information and is not applicable to many loci.

One of the most widely used approaches for detecting base substitutions in DNA is single-stranded conformational polymorphism (SSCP) analysis, whereby PCR amplified DNA is denatured and separated by electrophoresis on a non-denaturing polyacrylamide gel (5). Under these conditions the mobility of a single-stranded DNA fragment is dependent not only on length, but also its sequence. This technique can be used to detect the majority (~80%) of base substitutions in short (<1000 bp) fragments (5).

In an attempt to reproduce the SSCP effect for large DNA fragments we have analyzed isoalleles of the minisatellite MS32 (6). Individual MACH was previously identified as pseudohomozygous for ~2.5 kb MS32 alleles by Southern blot analysis, with alleles differing in length by one repeat unit (29 bp, 72 and 73 repeats) and by at least 31 nucleotide substitutions (as determined by MVR analysis (1)). These isoalleles were too large to be separated by standard polyacrylamide based SSCP (Katrina MacKay, personal communication). We therefore tested an agarose gel system instead. Isoalleles, either separate or combined, from this individual were PCR amplified and labelled on one or other strand, heat denatured and separated by electrophoresis on a non-denaturing agarose gel. Both alleles were clearly resolved (Figure 1). This procedure was also successfully applied to a second individual (AS89, ref. (1)) pseudohomozygous for two alleles of identical length (~2.5 kb), although differing by at least 23 nucleotide substitutions (data not shown). MS32 repeats show a marked strand bias in nucleotide content, with one strand comprising 45% C and the complementary strand only 17% C. Curiously, for both pairs of isoalleles the C-rich strand gave sharper, more discrete bands and clearly resolved the two pairs of isoalleles. In contrast, the G-rich strand produced less discrete, broad 'fuzzy' bands which did not resolve the isoalleles so clearly.

SSCP analysis of minisatellite isoalleles on agarose gels was also tested on genomic DNA. Six DNA samples were digested with MboI and separated by agarose gel electrophoresis, both with and without prior heat denaturation. MS32 alleles were detected by Southern blot hybridization with an MS32 repeat unit probe (6). Two of the DNA samples (CAWE and DAMO) were derived from MS32 heterozygotes and single-stranded analysis produced four bands (two sharp and two broad bands), two from each allele, as expected for both individuals (Figure 2). The pseudohomozygote MACH produced a four-band single-stranded pattern similar to that obtained by PCR-SSCP (Figure 2). Three other apparent homozygotes, with MS32 alleles too large to be efficiently amplified by PCR (5.2, 6.3 and 6.5 kb), were also analyzed. Two produced a three-band single-stranded pattern (two sharp and one broad) indicating heterozygosity for alleles distinguishable by genomic-SSCP. The third apparent

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than the G-rich strand. To be distinguishable by agarose-based SSCP analysis (data not shown), the products were purified by ammonium acetate/isopropanol precipitation and redissolved in 20 μl H₂O. Samples for single-stranded (ss) analysis were mixed 1:2 with formamide loading buffer (95% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue) and heat denatured at 80°C for 3 minutes prior to loading. 100 pg of each end-labelled allele was separated by electrophoresis through a 37cm 1.2% agarose gel in 1xTBE (89 mM Tris-borate, 2 mM EDTA, pH 8.3) at 150 V for 16 hours. After electrophoresis the gel was dried and autoradiographed overnight. Use of primer C1 allowed specific labelling of the G-rich strand, whilst primer D1 allowed specific labelling of the C-rich strand. Alleles were amplified from total genomic DNA (T) and from alleles separated by single molecule dilution of genomic DNA (A, B, ref. (1)).

homozygote produced only a two-band single-stranded pattern; subsequent MVR analysis (2) showed this person to be a true homozygote.

To test the generality of agarose gel—SSCP for analysing large alleles, we investigated two other minisatellites, MS205 and MS1 (7,6). Southern blot analysis of families with MS205 revealed one family in which both parents shared two pairs of alleles of the same length and for which the majority of children appeared homozygous (7). For this family both parents and all the children were typed by agarose-gel PCR-SSCP and the products detected by Southern blot hybridization. One of the two pairs of isoalleles could be resolved by SSCP and its Mendelian segregation was demonstrated, increasing the level of linkage data obtainable from this family (data not shown). Subsequent MVR mapping (8) has revealed that both pairs of isoalleles are in fact different. The longer (~3.3 kb), separable pair of isoalleles differ in their total number of repeat units (53 and 54) and at 16 MVR positions, whilst the shorter (both 42 repeats, ~2.6 kb), inseparable alleles showed only 3 MVR map differences (8). MS205 displays a similar level of nucleotide content strand bias as MS32, and strand-specific oligonucleotide hybridization was used to show that the informative band was again derived from the C-rich strand. In addition, three small PCR amplified isoalleles (~1.2 kb) of another minisatellite locus, MS1 (6,9), were also shown to be distinguishable by agarose-based SSCP analysis (data not shown). Once again, the C-rich strand yielded greater resolution than the G-rich strand.

All three minisatellite loci studied by agarose gel SSCP show an extreme strand bias in terms of band width and resolving power; in all cases the C-rich strand produced sharper bands of greater resolving power. In most cases identically-sized alleles were not separated by the G-rich strand, and closely-sized alleles were only poorly resolved. Presumably, these broader bands indicate the existence of a number of closely related, but different conformations, each with similar, but non-identical mobilities. G-rich sequences are known to be capable of forming atypical secondary structures, including quadruplexes, in vitro (11), and such complex conformations, possibly enhanced by the tandem repeat nature of minisatellites, may not be appropriate for agarose gel SSCP analysis.

The resolution limits of agarose-based SSCP are difficult to assess. Those isoalleles at MS32, MS205 and MS1 which were successfully resolved contain multiple MVR differences. The two indistinguishable MS205 isoalleles nevertheless showed three MVR sequence differences. SSCP analysis of 350 bp of unique sequence DNA flanking MS32, known to contain a base substitutional polymorphism (10), produced no detectable differences on agarose gels, whilst standard SSCP using PAGE detected the known base substitution and revealed a further one (10). It therefore seems likely that agarose gel—SSCP will be more effective at distinguishing alleles with many repeat sequence and/or small length differences, and may not be applicable to screening non-repeated genomic DNA.

We have demonstrated that sequence differences and/or small length differences can give rise to significant changes in single
stranded mobility of minisatellite isoalleles during agarose gel electrophoresis. This technique can be applied not only to PCR amplified alleles, but also to restriction-digested genomic DNA using a simple modification of standard DNA profiling procedures. SSCP analysis on agarose gels can be used to increase the allele-length heterozygosity of minisatellite loci, suggesting that it may be used to increase the amount of information derived from minisatellites in individualisation, pedigree and linkage analysis.

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