SHORT COMMUNICATION

Minisatellite "Isoallele" Discrimination in Pseudohomozygotes by Single Molecule PCR and Variant Repeat Mapping

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The D1S8 hypervariable minisatellite MS32 has a heterozygosity of 97.5% based on detectable differences in allele length using standard Southern blot analysis. It has previously been shown that the basic repeat unit is in itself variable and that this may be used to map the internal structure of an allele. This method has already been used to establish that alleles of the same length may have differing internal structures between nonrelated individuals. We now extend this approach to demonstrate that two apparently homozygous individuals are in fact heterozygotes. For each individual the two comigratory alleles were separated, without cloning, using single molecule dilution (SMD) of genomic DNA and recovery with PCR. Mapping of the variant repeat units revealed highly diverged internal structures and, for one individual, a size difference of one repeat unit (29 bp). SMD and PCR recovery provide an efficient system for separating comigratory alleles without prerequirement for knowledge of sequence differences.

Minisatellite or variable number tandem repeat (VNTR) loci have proven to be of considerable importance in a wide range of genetic applications, especially in the areas of individual identification (Jeffreys *et al.*, 1985) and gene mapping (Nakamura *et al.*, 1987). The primary reason for their widespread use as genetic markers is their often very high level of allele polymorphism and resulting heterozygosity (Wong *et al.*, 1987; Nakamura *et al.*, 1987). Allelic state is usually determined by the measurement of DNA fragment length estimated from Southern blot hybridization of genomic DNA. This system is however limited by the resolving power of agarose gel electrophoresis, not only for distinguishing small differences in allele sizes between individuals, which can lead to spurious departures from Hardy-Weinberg equilibrium (Devlin *et al.*, 1990), but also for discriminating between true and false homozygotes. However, allele length is not the only criterion by which minisatellite loci may be distinguished. DNA sequencing of human minisatellites has revealed repeat unit sequence variability at almost all loci so far investigated (see Jeffreys *et al.*, 1990). This additional level of polymorphism greatly extends the potential resolving power of minisatellite loci allowing alleles of similar, or even identical, size to be distinguished on the basis of internal structure.

The D1S8 minisatellite (MS32) comprises a 29-bp repeat unit and has a reported heterozygosity of 97.5% based on allele length (Wong *et al.*, 1987). Sequencing of D1S8 has revealed an A to G transition in approximately 70% of the repeat units, resulting in the presence or absence of a *Hae*III restriction site (Wong *et al.*, 1987). In contrast, all repeat units are cut by the restriction enzyme *Hin*fI. The location of variant repeat units can be mapped in alleles amplified by PCR. Partial digestion of end-labeled alleles with *Hin*fI followed by gel electrophoresis and autoradiography produces a continuous ladder of labeled DNA fragments from which the number of repeat units can be determined. Comparison of *Hae*III and *Hin*fI partial digests enables each repeat unit to be scored as to whether or not it is cleaved by *Hae*III. Internal maps, or minisatellite variant repeat (MVR) haplotypes, thus generated can be simply encoded as a binary string of repeats cleaved or not cleaved by *Hae*III (Jeffreys *et al.*, 1990). We have previously shown that alleles of the same length shared by unrelated individuals may have widely differing internal structures, suggesting relatively distant genealogical origins (Jeffreys *et al.*, 1990).

Screening of D1S8 across large panels of unrelated people revealed two individuals (AS89 and MACH, of Pakistani and Chinese descent, respectively) who by

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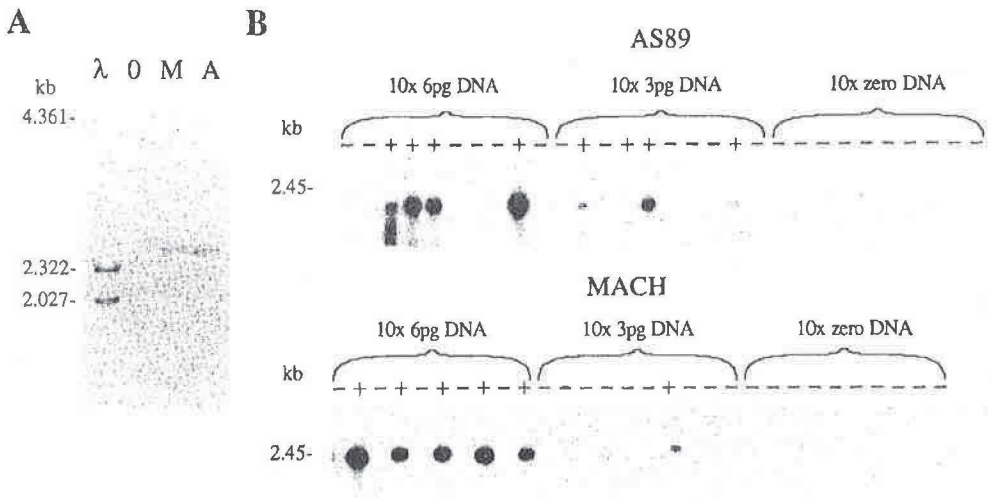

FIG. 1. Separation of minisatellite alleles in pseudohomozygotes by single molecule dilution and PCR. (A) D1S8 alleles amplified from total genomic DNA. For each individual 20 ng blood DNA was PCR amplified in a 10-μl reaction using the nested primers C1 and D corresponding to the flanking region of the D1S8 minisatellite (Ref. (3)). Amplified alleles were electrophoresed through a 1% agarose gel and visualized by ethidium bromide staining. Lanes: λ, λ *Hin*dIII marker DNA; 0, zero DNA negative control; M, amplified alleles from individual MACH; A, amplified alleles from individual AS89. (B) Allele separation by single molecule dilution. For each individual, genomic DNA was diluted in 5 m*M* Tris–HCl (pH 7.5) in the presence of 0.1 μ*M* PCR primers. Ten 10-μl PCR reactions containing either 6, 3, or 0 pg DNA were amplified for 28 cycles using the D1S8 minisatellite flanking primers A plus B. Products were detected by Southern blot hybridization. Primer sequences are given in Ref. (3). Derivatives C1 and D1 incorporate a 5′ extension containing an *Eco*RI restriction site that was used to generate end labeled PCR products.

*Sau*3AI restriction digestion and Southern blot analysis appeared to be homozygous for an approximately 2.5-kb allele at D1S8 (data not shown). PCR amplification of D1S8 from total genomic DNA revealed a single band on an ethidium bromide-stained gel for each individual (Fig. 1A). To determine if these individuals were indeed true homozygotes we attempted to map the internal structures of these alleles. Internal mapping from total genomic DNA produced an ambiguous autoradiograph with widely differing band intensities, presumably reflecting a composite internal map derived from two comigratory but discrete alleles (Fig. 2, lanes marked T). Since the alleles were inseparable by standard agarose gel electrophoresis, the individual alleles were separated by single molecule dilution (SMD) of genomic DNA (Jeffreys *et al.*, 1990; Ruano *et al.*, 1990) and recovery with PCR (Saiki *et al.*, 1988) using PCR primer pair A plus B, which correspond to the flanking sequence of the D1S8 minisatellite and allow amplification of the entire minisatellite allele (Jeffreys *et al.*, 1990). After 28 cycles of amplification a 5-μl aliquot was removed, electrophoresed, and Southern blot hybridized (Fig. 1B). Internal nested primers C plus D were used to reamplify the alleles from each of the presumptive single molecule-positive reactions up to a level visible on an ethidium-stained gel. All four alleles were completely mapped from both ends using DNA derived from at least three separate single molecule reactions for each allele (Fig. 2).

Individual AS89 was found to have two alleles of identical size, containing 71 repeat units, but of widely differing internal structures. One allele shares a 5′ MVR haplotype common to many individuals (unpublished data). The other allele belongs to another previously characterized set of homogeneous alleles composed almost entirely of repeats cleaved by *Hae*III. This individual is therefore a true heterozygote at this locus. Analysis of the second individual MACH, of Chinese origin, revealed this person to be a compound heterozygote at this locus, each allele having widely diverged internal structures and a length difference of 1 repeat unit (allele lengths 72 and 73 repeat units). Both alleles describe new 5′ haplotypes previously unseen in our studies on Caucasian individuals (Jeffreys *et al.*, 1990).

Our method for internal mapping does not involve cloning of single amplified molecules (minisatellites are frequently unstable on cloning in *Escherichia coli*), but samples the average properties of the entire amplified pool of PCR products. For each allele at least three separate single molecule amplifications were performed and in no case was any MVR map discrepancy found between them. As noted in previous experiments (Jeffreys *et al.*, 1990), it appears that *Taq* polymerase-induced misincorporation errors are not a problem with single molecule minisatellite mapping. Finally, in each case the superimposition of the two separated alleles gave rise to the same composite map as derived from total genomic

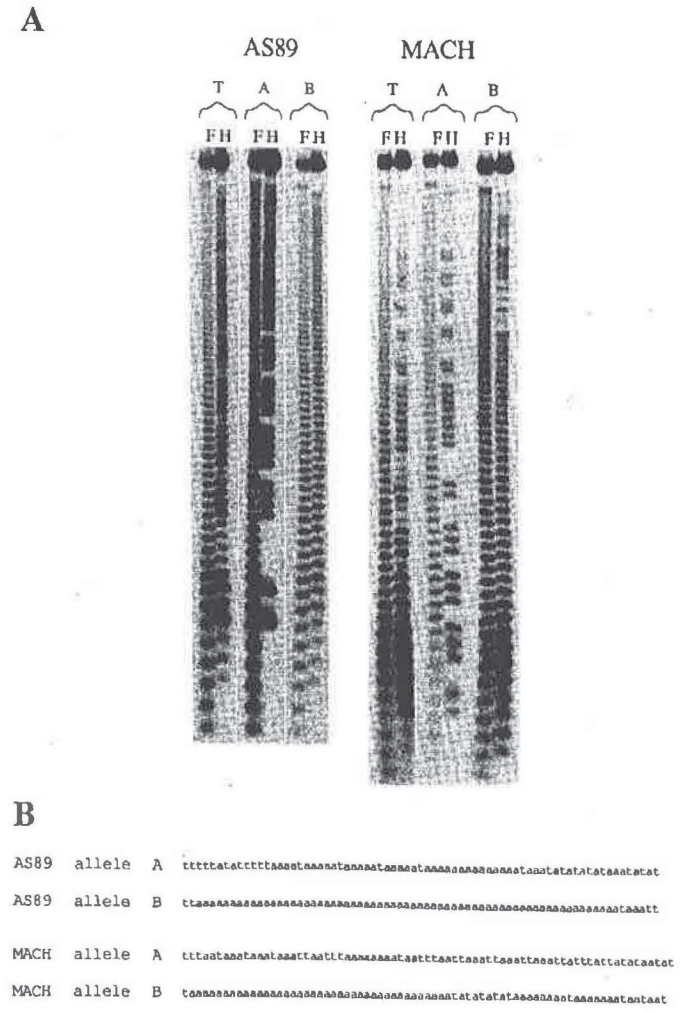

FIG. 2. Internal mapping of PCR-amplified D1S8 alleles. Internal mapping of all alleles was performed as previously described (3). (A) End-labeled products were partially digested with *Hin*fI (F) or *Hae*III (H), electrophoresed on a 1.2% agarose gel, dried, and autoradiographed. Lanes marked A and B are single molecule-derived alleles; lanes marked T are internal maps derived from total genomic DNA. (B) Encoded maps of all four single molecule-derived alleles, where A = repeat unit cleaved by *Hae*III and T = repeat unit not cleaved by *Hae*III.

DNA, confirming that the single molecule alleles recovered were the true alleles present in each individual.

Single molecule dilution and PCR recovery is a quick and efficient method for separating comigratory alleles of the same locus. Moreover it has several further advantages over cloning direct from a genomic library, requiring a very minimal quantity of input DNA, as well as a suitability for isolation of products unstable in a bacterial host. Likewise, it offers advantages over cloning from a PCR-derived library in that it has very low probability of incorporating polymerase errors and removes the potential problem of heteroduplex-derived chimeric "alleles." It also assumes no knowledge of internal sequence difference and thus allows isolation of uncharacterized polymorphisms.

Internal mapping of minisatellites greatly enhances the effective resolution of an already highly informative set of markers, as demonstrated here by its ability to distinguish pseudohomozygotes from true homozygotes. D1S8 is one of the few minisatellites for which an efficient internal mapping strategy has been developed so far, but work is in progress to increase the number of loci to which internal mapping can be applied. Internal mapping not only improves the general informativeness of minisatellite loci, but also provides important clues as to the evolution and generation of new alleles at these, some of the most invaluable, markers in the human genome.

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