Detection of β-thalassemia using an artifical-restriction fragment length polymorphism generated by the polymerase chain reaction

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By using the polymerase chain reaction (PCR) to introduce artificial-restriction fragment length polymorphisms (A-RFLP's) into amplification products of the human β-globin gene, we have developed a rapid and reliable DNA-based test for pre- or postnatal identification of the β^{39} allele associated with β-thalassemia (1). A-RFLP's are generated by introducing a single nucleotide mismatch into the 3′ end of an oligonucleotide primer which is itself positioned immediately adjacent to the polymorphic site in codon 39; this mismatch can cooperate with the amplified nucleotide sequence variation in the wild type and mutant β-globin alleles to generate specific restriction enzyme sites.

Figure 1 predicts that this 300 bp amplification product contains three RFLP's at the site of the β^{39} mutation. One of these sites, a StyI site, cannot be used for RFLP typing due to the presence of a closely occurring endogenous site. However, the artificial EcoRII site, generated by the PCR, and the endogenous polymorphic MaeI site, propagated by the PCR, can be used to identify wild type and mutant alleles respectively (Figure 1). Because our probe consisted of a mixture of B-39 and PCO3 primers, we performed a Southern analysis to eliminate primer hybridization signals which may arise in a standard 'dot blot' assay, owing to non-specific amplification.

Digesting each of the three DNA's with StyI results in PCR products which lack both primer binding sites as shown by lack of hybridization in lanes B, F, and J. MaeI digestion of β^{39}/β^{39} DNA is also predicted to generate a fragment which lacks both primer binding sites since a polymorphic MaeI site is present in the mutant allele and an endogenous site is situated just inside primer PCO3. As predicted, EcoRII digestion does not remove primer binding sites (compare lanes **A** and **C**). Digesting wild type DNA with either MaeI or EcoRII releases one half of the available primer binding sites (compare lanes **K** and **L** to **I**). Similarly, digestion of heterozygous DNA yields a unique primer hybridization pattern. MaeI digestion removes three of the four available primer binding sites, whereas EcoRII digestion removes one quarter of these sites (lanes E−H).

From a population of 25 point mutations (1), which cannot be identified through a natural RFLP analysis, we found that A-RFLP's could be produced for 23/25 β-thalassemia mutations using primers containing only one nucleotide mismatch (3) (including β^{IVS1-1} and $\beta^{IVS1-110}$), while the remaining mutations required two mismatches (2) to generate an A-RFLP. Our anti-hybridization strategy allows genotyping of large PCR fragments (unpublished results) and provides an avenue for the selective cloning of individual alleles from an amplification reaction by utilizing specific vector cloning sites.

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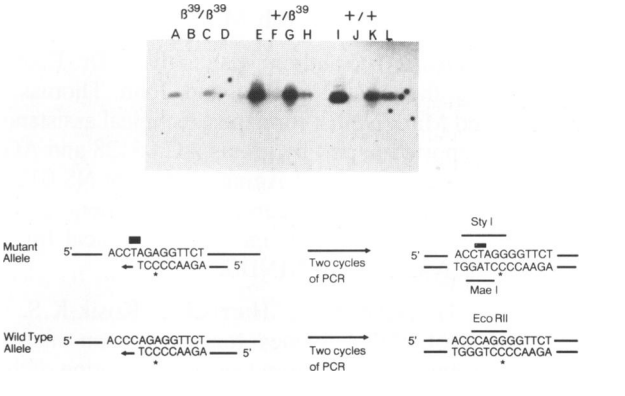

Figure 1. Southern analysis of amplified genomic DNA. Lanes A to D, E to H, and I to L represent DNA amplified from β^{39}/β^{39}, + β^{39}, and +/+ genomic DNA respectively. The individual lanes represent amplified DNA that was mock digested (A,E,I), or treated with StyI (B,F,J), EcoRII (C,G,K), or MaeI (D,H,L) and probed with a mixture of B-39 and PCO3 primers. Amplification of genomic DNA was performed as described (2) using the primers PCO3 (4) and B-39 (5′:C-AAAGGACTCAAAGAACCCCT) and cycle times of 2 min at 94°C, 2 min at 55°C and 3 min at 72°C. Two μl aliquots of the amplified DNA were then subjected to restriction enzyme digestion, and electrophoresed through 0.8% agarose gels in TAE buffer and transferred to Hybond N membranes (Amersham). Oligonucleotide primers (40 ηg each) were mixed, phosphorylated, and hybridized to the membranes according to the manufacturer.