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SHORT REPORT

Intron 22-specific long PCR for the Xba I polymorphism in the factor VIII gene

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Summary. The Xba I polymorphic site in the factor VIII gene is present in the int22h-1 region which is found in two other copies (int22h-2 and int22h-3) distal to the gene. Previously the polymorphic status of the Xba I locus was studied by either Southern blot or PCR that amplified all three copies. Here we report the use of a long PCR that specifically amplifies the intragenic site in intron 22, making use of this marker an easy and reliable assay. Moreover, about 25% of previously uninformative Turkish haemophilia A families examined with three markers proved to be informative for linkage analysis, using the Xba I polymorphism.

Keywords: factor VIII, Xba I polymorphism, long PCR, haplotype analysis, molecular diagnosis.

Haemophilia A is one of the most common bleeding disorders in man, affecting 1 in 5000 male births. It is caused by mutations in the factor VIII gene, of which an inversion mutation accounts for about 45% of the severe cases (Lakish *et al*, 1993). Recently a long PCR assay has been developed for the detection of this inversion that allows accurate prenatal diagnosis in about 25% of all haemophilia A cases (Liu *et al*, 1998). Other causative mutations are distributed all over the 9·1 kb coding region, thus making their identification a very labourious procedure. Therefore, prenatal diagnosis and carrier identification is still largely based on following the segregation of the affected chromosome by linkage analysis rather than direct identification of the causative mutation. Linkage analysis largely depends on the availability of informative markers.

One such marker is the Xba I polymorphism which is located in intron 22, int22h-1 region (intron 22 homologous region 1) that has two other copies (int22h-2 and int22h-3) distal to the factor VIII gene (Naylor *et al*, 1995). This diallelic marker is found to be informative in 48% of the cases among Caucasians when studied by Southern blot method, which can differentiate the intragenic from the extragenic sites (Wion *et al*, 1986). The heterozygosity in the XbaI locus is also about 50% in most other populations (Aseev *et al*, 1994; Chan *et al*, 1998; Kogan *et al*, 1987; Van de Water *et al*, 1991). Southern analysis is labour-intensive, and unless the gel is run for high resolution it may result in misdiagnosis due to the confusion of the extragenic copies with the intragenic one (Van de Water *et al*, 1994). A PCR assay of the XbaI polymorphism has also been described but it amplifies all three copies of the polymorphic site, thus making the PCR of limited use in carrier detection and prenatal diagnosis (Kogan *et al*, 1987). Consequently, these drawbacks in the detection methods have often neglected the use of the Xba I marker in linkage studies.

Recently, Liu *et al* (1998) reported additional DNA sequences in the intron 22 (int22h-1) region that allowed the amplification of factor VIII-intron 22-specific sequences in the int22h-1 region. Here we report for the first time the use of an intron 22-specific long PCR for the detection of Xba I polymorphism. This will enable the determination of the heterozygote state in females and thereby increase the number of informative markers available for haplotype analysis, carrier detection and prenatal diagnosis in families afflicted with haemophilia A.

MATERIALS AND METHODS

DNA samples. DNA was extracted from the peripheral leucocytes of haemophilia A probands and their families or from normal individuals according to standard procedures.

Amplification reaction. PCR was done in 25 μl total volume containing 200 ng genomic DNA, 2·5 μl of Buffer 2 (Expand PCR system, Boehringer Mannheim, Mannheim, Germany),

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7·5% DMSO (dimethyl sulphoxide), 250 μmol/l each of dGTP and 7-deaza-dGTP (Boehringer Mannheim, Mannheim, Germany), 500 μmol/l from other dNTPs, 1 μl of Expand Long Template DNA polymerase, 0·4 μmol/l of each primer. Forward primer (F): GCC CTG CCT GTC CAT TAC ACT GAT GAC ATT ATG CTG AC, reverse primer (R): TTC AAC ACG ACC ACC ATC TCT CAA GTG GCC. F is the primer P as reported by Liu *et al* (1998) and R was designed for specific amplification of the XbaI variable site in int22h-1.

PCR cycling was performed in a GeneAmp 2400 thermocycler (from Perkin Elmer), using thin-wall tubes, as follows: 94°C for 2 min followed by 30 cycles of 94°C for 12 s, 65°C for 30 s, 60°C for 7 min with auto-extension of 20 s for the last 20 cycles, followed by 15 min at 68°C. About 5 μl of the PCR product was run on 1% agarose gel to check for the presence of the desired product.

Xba I digestion and electrophoresis. Digestion was done in a total volume of 12 μl containing: 10 μl of PCR product, 10 units of Xba I enzyme (from Promega), 1·2 μl of Xba I restriction buffer (buffer D) and bovine serum albumin to a final concentration of 0·1 mg/ml. The digestion reaction was kept at 37°C overnight. The digestion product was run on a 20 cm 1% agarose gel at 100 V for about 5 h or until the optimum band separation was achieved. The gel was stained with ethidium bromide and the bands were visualized by exposure to UV light.

RESULTS

A specific PCR assay that amplified the factor VIII-intron 22 copy of the Xba I polymorphism was developed using the primers and conditions described in the Materials and Methods section. Fig 1A shows schematically the position of the primers used and XbaI and KpnI sites relative to the Int22h 1 repeat in intron 22. The amplification product is 6·2 kb in size and contains an invariable XbaI site. Digestion of the PCR product with XbaI resulted in the production of 0·7 kb, 4·8 kb and 0·65 kb fragments in genotypically (+) hemizygous males and homozygous females, and 5·4 kb and 0·7 kb fragments in genotypically (−) hemizygous males and homozygous females (Fig 1B). Heterozygous females (+/− genotype) had both 5·4 and 4·8 kb fragments.

The XbaI polymorphism in the F.VIII gene was also examined by Southern analysis in the Turkish population. The intragenic Xba I site was positive in 32 and negative in 31 male individuals, resulting in an expected heterozygosity of 50% among the Turkish population. Interestingly, both of the extragenic copies were found to be Xba I positive in only one sample; all other samples were Xba I negative in both extragenic copies. The individual with a (+) Xba I genotype in extragenic copies also had the (+) genotype for the intragenic copy.

Twelve females previously not informative for linkage analysis with the intragenic markers (Hind III and microsatellite repeats) were also analysed for the intragenic XbaI polymorphism by long PCR and three were found to be informative, suggesting that the inclusion of the XbaI polymorphism would contribute to a further increase in the number of informative females in the population, for possible linkage analysis.

The Xba I PCR assay described here was reproducible and was in complete concordance with the Southern blot method in all samples studied.

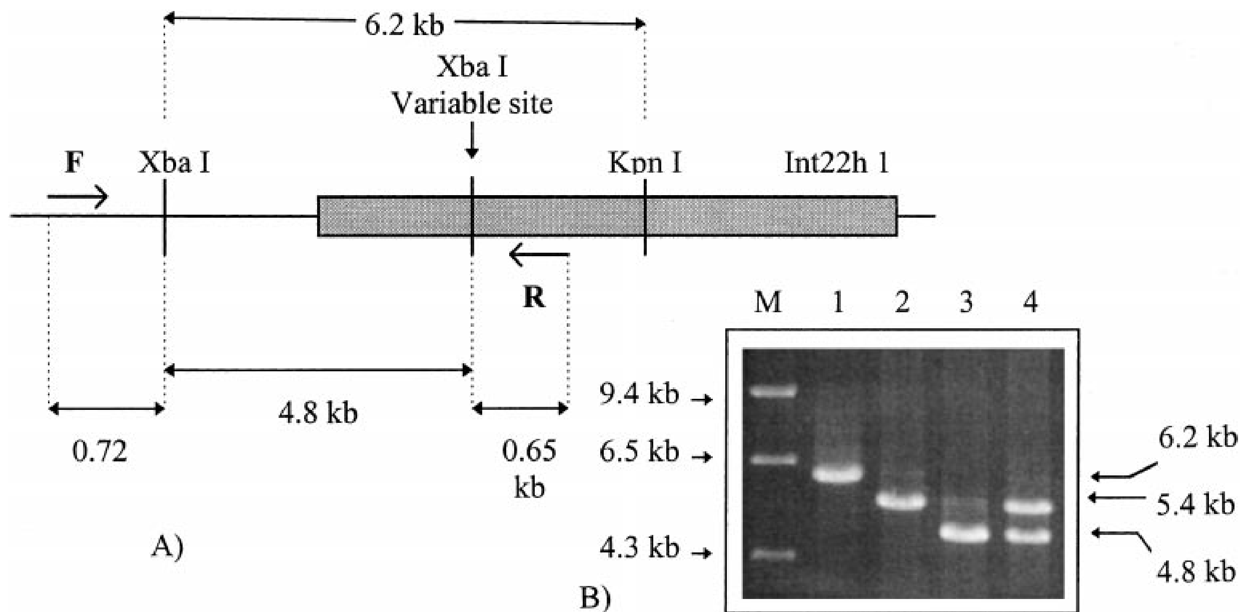

Fig 1. Intron 22-specific long PCR for the XbaI polymorphism. (A) Schematic diagram of the intron 22 region showing the relative positions of the primers for Xba I long PCR, and the restriction enzyme map of Xba I and Kpn I in the region. (B) Agarose gel electrophoresis of the long PCR and Xba I digestion products for all possible genotypes. Lanes M: λ/hind III marker; 1: undigested product; 2: male (−) or homozygous female (−/−); 3: male (+) or homozygous female (+/+); 4: heterozygote female (−/+).

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DISCUSSION

Due to the large size of the factor VIII gene and the absence of major mutation hot spots, except for the intron 22 inversion mutation, molecular diagnosis in haemophilia A is still largely based on haplotype analysis. Many polymorphic markers are currently in use in linkage analysis. The microsatellite repeats in introns 13 and 22, Hind III and Bcl 1 markers in introns 19 and 23, respectively, are among the most commonly used polymorphic markers in the factor VIII gene. The simultaneous use of microsatellite markers and Hind III was found to be informative in only 66% of the cases among the Turkish haemophilia A families (Jarjanazi *et al*, 1998). Therefore the additional use of any highly polymorphic intragenic marker(s) would considerably increase the informativeness among these families.

In this study, we report the development of a long PCR-based method for the detection of the intron 22 Xba I polymorphism without interference from the extragenic copies of this region. The XbaI long PCR assay is easy to perform, is highly reproducible, and eliminates the need for the more laborious and costly Southern blot analysis of the polymorphic site.

Using the long PCR based Xba I assay, we have shown that, at least in the Turkish population, about 25% of previously non-informative females were eligible for molecular diagnosis. This assay is also expected to contribute to factor VIII linkage analysis in other populations since the polymorphism has a heterozygosity rate of about 50% in almost all populations examined.

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