The Malmö Polymorphism of Factor IX: Establishing the Genotypes by Rapid Analysis of DNA

By John B. Graham, Glenna R. Kunkel, Gary S. Tennyson, Susan T. Lord, and Dana M. Fowlkes

A DNA polymorphism in the coding region of coagulation factor IX—potentially valuable for carrier detection, prenatal diagnosis, and population studies—was described in 1985. It had been discovered with monoclonal antibodies that distinguish between threonine and alanine as the 148th residue of the peptide. Its use as a diagnostic tool has been limited because threonine-containing factor IX (Malmö A) is dominant to alanine-containing factor IX (Malmö B) in immunossays of plasma; therefore, detection of Malmö heterozygotes is not possible in all instances. A DNA method for recognizing all heterozygotes has been developed, but it also has limitations. We report the development of another DNA procedure based on amplification of the relevant DNA with the polymerase chain reaction (PCR). This method is quick, avoids the use of isotopes and x-ray film, and specifically identifies all the Malmö genotypes: hemizygotes, homozygotes, and heterozygotes. The procedure can be performed satisfactorily on small samples of blood (<1 mL) as suggested by Kogan et al (N Engl J Med 317:885, 1987). The method described is applicable to any genetic polymorphism that overlaps a restriction enzyme recognition site.

MATERIALS AND METHODS

Blood was obtained from volunteers in accordance with the university’s policies on informed consent under permission no. 72 PATH 34 of the Committee on Human Rights. Nuclear pellets and DNA were prepared as described by Graham et al and Lubahn et al. DNA was also prepared from small samples of whole blood as described by Kogan et al.

Oligonucleotide primers were synthesized on an Applied Biosystems 380-A Synthesizer (Applied Biosystems, Foster City, CA) using reagents from American Bionetics (Hayward, CA). Primers were purified using triply on reverse phase HPLC. The 27 base primers for production of the 407 bp amplification product were:

Forward sequencing:

5' GATTGAAAACGTCATGAAAAATAC 3'

Reverse sequencing:

3' GGTCCAGTATAGGAACTGCTGATGG 5'

PCR was performed in two ways. Earlier, a computer-controlled, cycling convection air oven was used in a modification of the method of Kogan et al. A 12-minute temperature cycle consisted of: three minutes from 70°C to 90°C for denaturation, 4 T/2 minutes from 90°C to 59°C for annealing, 1 4 minutes from 59°C to 70°C, then three minutes at 70°C for extension. Since acceptable results were not consistently obtained and the cycles were long, we turned to a Programmable Cyclic Reactor (Ericomp, San Diego) in a 4 1/2-minute cycle at two temperatures, 60°C for four minutes and 90°C for 30 seconds. Suitable results have been obtained consistently.

Three units of Taq polymerase were added in a single aliquot (Cetus, Norwalk, CT) after 1 to 3 μg of genomic DNA was denatured for ten minutes at 90°C. The reaction mixture, which was overlain with mineral oil to minimize evaporation, was essentially the same as that of Kogan et al except that 2.5 mg/mL of gelatin was substituted for 170 μg/mL of bovine serum albumin (BSA) and the Mg2+ was 4.5 μmol/L. After 30 cycles the reaction mixture was concentrated to half volume in a Speedvac (Savant, Hicksville, NY), precipitated with 95% ethanol and oyster glycogen, and washed with 70% ethanol. The sample was dried in the Speedvac, reconstituted in 40 μL of TE buffer, and an aliquot of 5 μL run (about 1 hour) in a 6% polyacrylamide mini-gel at 100 V to assure that amplification occurred. Then, 7 μL of the reconstituted mixture containing the amplification product was digested with 3 U MnlI (New England Biolabs, Beverly, MA) diluted in 1IB buffer A. The digested sample and an undigested sample were then run in adjacent wells in 6% polyacrylamide for 1 to 1 1/2 hours, stained with ethidium bromide and photographed under UV light.

RESULTS

Figure 1 is a diagram of a portion of Exon 6 of the factor IX gene, which contains the coding region for the activation...
peptide and two recognition sites for MnlI. The relationships between the DNA, peptide, and immunologic phenotypes are shown.

The application of the method to the five possible Malmö genotypes is shown in Fig 2. A 126-bp fragment is always present in digestion lanes, because of the invariance of the first MnlI site, and it serves as an internal control of digestion. When the second site is absent (A2, AA2), there is also a 279-bp fragment. When the second site is present (B2, BB2), there are three fragments: 126, 159, and 279 bp. (The 126 and 120 bp fragments do not resolve, running as a slightly broader, somewhat “fuzzy” band.) When both sites are present (AB women) there are easily distinguishable 279 and 159 bands.

Figure 3 shows the Malmö genotyping of one normal and two hemophilia B families. Figure 3A represents the normal kindred, and the DNA test showed that both the grandmother and the granddaughter are AB heterozygotes. However, their monoclonal antibody (MAB) 9.9:9.1 ratios, shown below the figures, gave very different results. The granddaughter, with a ratio of .09, could be classified as AB immunologically, but the grandmother with the 1.07 ratio would be classified as normal. The hemizygous Malmö B father is documented by his zero 9.9:9.1 ratio.

Figure 3B shows a small family transmitting hemophilia B in which the DNA method was informative, because the mother was heterozygous for both hemophilia B and the Malmö dimorphism. Linkage of the hemophilia gene to the Malmö A allele in the mother means that the AA sister is a carrier of hemophilia B.

Figure 3C shows an uninformative result in a hemophilia B kindred in which the mother was homozygous for the Malmö polymorphism. When tested for the Bam HI polymorphism, for which the mother was heterozygous, the daughter was found to be normal.

Table 1 shows application of the method to the population genetics concerning two ethnically distinct groups of normal women. The Anglo women are 21 female medical students at the University of North Carolina, all of whose American ancestors are believed to have come from the British Isles. The Basque women are residents of Boise, Idaho, all of whom had four grandparents who were of Spanish Basque origin. Gene frequencies for each group, determined by testing an equal number of men of each group immunologically (un-
Fig 3. Malmö polymorphism. (A) The immunoassays and DNA results of a family of normal persons are shown. Genotypes (DNA) are shown inside the figures. The number below each figure is the ratio of IX:Ag determined by MoAbs 9.9 (numerator) and 9.1 (denominator). MAB 9.9 recognizes only Malmö A, while 9.1 recognizes all factor IX molecules. Elsewhere it has been shown that this ratio is currently the best statistic for predicting AB heterozygosity in type A women. Mean ratios in that study were: AA = 98 (range, 1.36–48); AB = 67 (range, 1.47–22). Variances were large in both groups and 50% of AB women had ratios within 2 SD of the normal mean (ratio was 1.13 for A men, and 0 for B men). (B, C) H, hemophiliac man; D, normal man; O, hemophilia carrier; S, possible hemophilia carrier.

Pubished data (May 1988), were used to calculate the expected genotype frequencies in the women. The observed genotype numbers for each ethnic group were very close to those expected and were not significantly different from expected in either population. In addition, the Anglo and Basque gene frequencies were not significantly different.

DISCUSSION

The recognition of the Malmö polymorphism in factor IX molecules by MoAbs has great potential for carrier detection and prenatal diagnosis in hemophilia B, and in population studies of factor IX. Graham et al have reported that Malmö, the first polymorphism discovered in the coding region of the factor IX gene, was more frequently heterozygous in a sample of Swedish women than the XmnI and TaqI RFLPs, which are widely used, and that Malmö alone is almost as useful as the three together (50% vs 59%, respectively). This suggests that a simple test for the polymorphism would be very useful clinically.

Large numbers of plasma samples can be studied for the polymorphism immunologically at relatively low cost, and the results from men (in whom phenotype = genotype) may be used to dependably estimate gene frequencies in populations. Immunoassays have the further advantage that plasma samples are easily collected, transported, and stored.

The dominance in plasma of Malmö A over Malmö B and the phenomenon of “lyonization” make the immunologic distinction between AA and AB women problematic (Fig 3A). In addition, immunoassays cannot be applied to fetal samples before the 12th week of gestation. A different approach, such as a DNA method, is needed. The method used by Winship and Brownlee—Southern blotting of genomic DNA hybridized to labeled synthetic oligomers in dried gels—is sufficient but impractical. Disadvantages include weak signals that may require long exposures and lead to long delays, even repetition, and the necessity to use radioisotopes and x-ray film in Southern blotting.

Amplification of the relevant DNA by the PCR, and determination of the presence or absence of an MnlI restriction site by ethidium bromide staining alleviates the cost and labor, solves the problem of weak signal, and avoids the use of radioisotopes and film. Furthermore, it is applicable to the DNA of men, women, and fetuses. Identification of the grandmother in Fig 3A as an AB type whose immunoassays were in the normal range emphasizes its usefulness. Figures 3B and C show that the PCR method is not always applicable, the corollary of 50% heterozygosity being 50% homozygosity. Genotyping was successful with the Bam HI polymorphism, a restriction fragment length polymorphism (RFLP) much more apt to be useful than the commonly used Taq I and Xmn I polymorphisms, which are in strong linkage disequilibrium with Malmö. The Bam HI RFLP has now been adapted to a PCR procedure by Zhang et al and ourselves (unpublished data, January 1989), which will greatly improve the likelihood of finding a doubly heterozygous mother in a hemophilia B family.

Beginning with DNA, a single technician in our laboratory is able to examine about 40 samples a week efficiently, in overlapping batches of 10. This volume approaches that used on the DNA of small blood samples obtained by finger prick or venipuncture. The ease of the procedure and the quality of the results, its applicability to carrier detection and prenatal diagnosis, and the avoidance of radioisotopes and x-ray film suggest that many laboratories may wish to adopt it as their standard clinical method for evaluating hemophilia B families. The method should remain useful for these purposes until “family-specific” DNA methods eventually supplant methods using linkage to RFLPs. We might have proceeded differently had we not preferred to distance ourselves from the use of isotopes. We might have prob the amplification product with radiolabeled oligonucleotides and observed the presence or absence of hybridization in the manner of Kogan et al.

The PCR method is also a superb tool for population studies on X-linked polymorphisms such as Malmö. Only men can be sampled immunologically without ambiguity, but all genotypes can be assessed with the PCR method. This

Table 1. Frequency of Malmö Genotypes in Women

<table>
<thead>
<tr>
<th></th>
<th>Anglos</th>
<th>Basques</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expected*</td>
<td>Observed</td>
</tr>
<tr>
<td>AA</td>
<td>8.6</td>
<td>8</td>
</tr>
<tr>
<td>AB</td>
<td>9.7</td>
<td>10</td>
</tr>
<tr>
<td>BB</td>
<td>2.7</td>
<td>3†</td>
</tr>
<tr>
<td>T</td>
<td>21</td>
<td>21</td>
</tr>
</tbody>
</table>

†Determined by PCR reaction.
‡A null genotype determined by immunoassay.
RAPID DNA ANALYSIS OF COAGULANT F IX

greatly increases the available information, since each woman provides twice as much information as each man. Since very small samples of blood are adequate, we hope to refine the method to the point where several drops of blood on filter paper are sufficient to classify an individual for the polymorphism. This would greatly simplify field work, especially in remote locales. It might even become possible for local practitioners to collect the samples on filter paper and send them by ordinary mail to a central laboratory for analysis.

Finally, we recommend use of the described procedure for the clinical and genetic study of any DNA polymorphism whose DNA sequence overlaps that of a restriction enzyme recognition site.

REFERENCES

The Malmo polymorphism of factor IX: establishing the genotypes by rapid analysis of DNA

JB Graham, GR Kunkel, GS Tennyson, ST Lord and DM Fowlkes