Hemophilia A: Carrier Detection and Prenatal Diagnosis by DNA Analysis

By Mario Pecorara, Lucia Casarino, Pier Giorgio Mori, Massimo Morfini, Giuseppe Mancuso, Angela Maria Scrivano, Elio Boeri, Angelo Claudio Molinari, Raffaello De Biasi, Nicola Ciavarella, Franco Bencivelli, Teo Ripa, Guido Barbujani, Angela Loi, Lucia Perseu, Antonio Cao, and Mario Pirastu

In this study, we used DNA polymorphisms for carrier detection and prenatal diagnosis of hemophilia A in a large group of Italian families. The restriction fragment length polymorphisms (RFLPs) investigated were the intragenic polymorphic BclI site within the factor VIII gene; the extragenic multiallelic TaqI system at the St14 locus; and the extragenic BglII site at the DX13 locus. The factor VIII probe was informative in 30%, St14 in 82%, and DX13 in 60% of obligate carriers. The combination of factor VIII-BclI and St14-TaqI showed that 91% of obligate carriers were heterozygotes for one or both; with all three probes, only 4% of obligate carriers were noninformative. In families clearly segregating for hemophilia A, RFLP analysis allowed us to define the carrier status for the hemophilia A gene in all 27 women tested. RFLP analysis allowed us to exclude the carrier status in 39 of 45 female relatives of sporadic patients. The combination of RFLP analysis and biological assay of factor VIII allowed us to identify a de novo mutation in the maternal grandfather in 7 of 12 of the families with sporadic cases, for which members of three generations were available for study. Nine of 10 couples requesting prenatal diagnosis provided informative RFLP DNA pattern. Carrier status was excluded in two women, two fetuses were shown to be female, and prenatal diagnosis was carried out in five pregnancies by DNA analysis. Prenatal testing was successful in three instances and failed in two because a sufficient amount of chorionic villous DNA was not obtained for the analysis.

Hemophilia A is the most common human bleeding disorder, affecting ~1 male in 10,000. It results from a deficiency or abnormality of clotting factor VIII-C and is inherited as a X-linked trait.1 The locus for factor VIII has been assigned to band q28 of chromosome X.2 Until a few years ago, carrier detection was based on estimation of the ratio of factor VIII coagulant activity (FVIII-C) to von Willebrand factor antigen (vWF:Ag), which is accurate in 80% of cases.34 Prenatal diagnosis has been carried out by conventional analysis of fetal blood obtained at 18 to 20 weeks of gestation by fetoscopy,5 a procedure associated with a high risk of fetal mortality. Recently, the factor VIII gene was cloned6-11 and three12 DNA polymorphisms within the factor VIII-C were detected.12-14 In addition, random X chromosome-specific DNA probes, known DX1315 and St14,16 closely linked to the factor VIII gene, detected several restriction fragment length polymorphisms (RFLPs). These discoveries have been applied to carrier detection12,17-20 and prenatal diagnosis of hemophilia A.21-22 The intragenic polymorphisms were informative only in a limited number of the families investigated, whereas the DX13 and, less frequently, the St14 probes showed recombination with the factor VIII locus.17,18,23-25

In this article, we describe the use of a factor VIII cDNA probe in combination with X chromosome-specific random probes, DX13 and ST14, for carrier detection and prenatal diagnosis of hemophilia A in a large series of families of Italian descent.

MATERIALS AND METHODS

Subjects. After informed consent was obtained, blood samples were collected from members of 61 families followed at the hemophilia center of Gaslini Institute in Genoa, the Pediatric Clinic in Palermo, the hematology department of Carreggi Hospital in Florence, and the 2nd medical clinic and the hemophilia center of Pellegrini Hospital in Naples.

Coagulation and immunologic assays. Factor VIII coagulant activity (VIII-C) was measured by a one-stage method; vWF:Ag was determined by a modification of Laurell’s technique.25

DNA analysis. Venous blood samples were collected into citrate, and the DNA was extracted from WBCs by standard techniques.26 Chorionic villous samples were obtained by the transcervical route under ultrasound guidance using a malleable silver cannula.27 The chorionic villous samples were checked microscopically to exclude contamination with maternal decidua before being dissolved in 10 mmol/L of Tris-HCl (hydroxymethylaminomethane hydrochloride) pH 7.5, 1 mmol/L of EDTA, 2% sodium dodecyl sulfate (SDS), 8 mol/L of urea, and 0.3 mol/L of NaCl. This was followed by extraction with phenol and then chloroform. The DNA was then precipitated with ethanol and reconstituted in TE buffer (10 mmol/L of Tris-HCl pH 7.5, 1 mmol/L of EDTA), under conditions recommended by the manufacturers. Resulting fragments were separated by electrophoresis on a 0.8% agarose gel and transferred to nitrocellulose filters by Southern blotting.28 Bound DNA was hybridized with probes radiolabeled with 32P by nick-translation.29 Hybridization was performed overnight at 42 ℃ in a solution containing 50% formamide, 50 mmol/L of Hepes, pH 8.0, 5 x Denhardt’s [1 x = 0.2% bovine serum albumin (BSA), 0.2% polyvinyl-pyrrolidone, 0.2% Ficoll 400], 6 x 1 x 0.15 mol/L of sodium chloride and 0.015 mol/L of sodium citrate (SSC), dissolved in 10 mmol/L of Tris-HCl pH 7.5, 1 mmol/L of EDTA and 2% sodium dodecyl sulfate (SDS), then washed with 2 x SSC, and autoradiographed.

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The combination of factor VIII-BclI and St14-TaqI showed that 91% of obligate carriers were heterozygotes for one or both; with all three probes, only 4% of obligate carriers were noninformative. In families clearly segregating for hemophilia A, RFLP analysis allowed us to define the carrier status for the hemophilia A gene in all 27 women tested. RFLP analysis allowed us to exclude the carrier status in 39 of 45 female relatives of sporadic patients. The combination of RFLF analysis and biological assay of factor VIII allowed us to identify a de novo mutation in the maternal grandfather in 7 of 12 of the families with sporadic cases, for which members of three generations were available for study. Nine of 10 couples requesting prenatal diagnosis provided informative RFLP DNA pattern. Carrier status was excluded in two women, two fetuses were shown to be female, and prenatal diagnosis was carried out in five pregnancies by DNA analysis. Prenatal testing was successful in three instances and failed in two because a sufficient amount of chorionic villous DNA was not obtained for the analysis.

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200 μg of salmon sperm DNA, 10% dextran sulfate and 4 × 10⁶ cpm/mL of heat-denatured probe. After hybridization, excess probe was washed off in 2 × SSC for 30 minutes at room temperature, followed by 1.5 hours at 50 °C in 0.1 × SSC and 0.1% sodium dodecyl sulfate (SDS). DNA bands that hybridized to the 3²P-labeled probe were detected by autoradiography with x-ray film (X-Omat, AR; Kodak) on intensifying screens (Cronex, Du Pont, UK).

DNA probes used were the 1.3-kilobase (kb) fragment of factor VIII cDNA covering exons 14 through 19 (kindly provided by Chiron Corporation, Emeryville, CA)²; DX13, a cloned sequence localized to band Xq28 by somatic cell hybrids³ and by in situ hybridization¹¹ (kindly provided by Dr K. E. Davies); and St14, a 3-kb DNA fragment originating from the q26-qter region of the X chromosome¹⁰ (kindly provided by Dr J. L. Mandel).

RESULTS

Frequency of intragenic and extragenic polymorphisms. We analyzed 61 hemophiliac DNA samples from unrelated families and 350 DNA samples from their relatives for the presence of a common BclI polymorphism within the factor VIII gene detected with a probe for exons 14 through 19, as well as for two common X chromosome RFLPs, which are detected by anonymous DNA sequences referred to as DX13 and St14, closely linked to the factor VIII gene. Typical Southern blotting analysis and RFLPs are shown in Fig 1. The BclI polymorphism 3’ to the exon 18 of factor VIII gene yields a 879-base pair (bp) fragment when the site is present and 1,185 bp when the site is absent. The St14 probe hybridized to human DNA digested with Taq I reveals polymorphic patterns with at least ten allelic fragments in addition to fragments of constant size,¹⁶,¹⁸ The DX13 probe hybridized to human DNA digested with BglI detects a RFLP with allelic DNA fragments of 5.8 and 2.8 kb in length. The frequencies of the allelic DNA fragments at the three polymorphic systems investigated are summarized in Table 1. Twenty-seven obligate carriers from families with at least two affected children in separate sibships were also investigated to define the proportion who were heterozygous for at least one polymorphism and in whom the linkage phase between the allelic DNA fragments and the hemophilia A locus was detectable (informative polymorphism). The factor VIII probe was informative in 9 of 27 (30%), St14 was informative in 19 of 23 (82%), and DX13 was informative in 14 of 23 (60%). Some subjects showed heterozygosity for more than one RFLP. When the factor VIII and St 14 probe were combined, 22 of 24 (91%) obligate carriers were heterozygous for one or both polymorphisms. When the three probes were combined, 23 of 24 (96%) carriers were informative. Eighteen women, homozygous at the BclI site, were also analyzed for the intragenic BglI polymorphism.¹¹ In none of them was this polymorphic site informative (data not shown).

Linkage of factor VIII to other markers. The linkage of St14 and DX13 loci to the BclI polymorphism revealed by the factor VIII probe was investigated in 20 and 19 families, respectively (Table 2). The number of meiotic events analyzed was 36 for the St14 locus and 31 for DX13. No recombination was observed between the St14 and factor VIII loci. The lod score for linkage of factor VIII to St14 is 4.8 at a recombination fraction of 0.0. This indicates that factor VIII gene is <6.4 recombination units with 95% confidence limits from the St14 locus. One recombinant was observed between DX13 and factor VIII/St14 loci (Fig 2). The lod score for linkage of factor VIII to DX13 is 2.1 at a recombination fraction of 0.045, indicating that factor VIII gene is <17.6 recombination units, with 95% confidence limits from DX13 locus.

Carrier detection. Carrier detection (Table 3) was carried out in 27 families with at least two hemophiliac males in

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**Table 1. Frequencies of Allelic DNA Fragments at Three Polymorphic Systems Intra (BclI/FVIII) and Extra (TaqI/St14, BglII/DX13) the Factor VIII Gene**

<table>
<thead>
<tr>
<th>Restriction Enzyme/Probe</th>
<th>BclI/FVIII</th>
<th>TaqI/St14</th>
<th>BglII/DX13</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of X chromosomes studied</td>
<td>153</td>
<td>173</td>
<td>156</td>
</tr>
<tr>
<td>alleles in kb/%</td>
<td>1.1/35</td>
<td>6.6/0.6</td>
<td>5.8/43</td>
</tr>
<tr>
<td></td>
<td>0.8/65</td>
<td>5.3/4.6</td>
<td>2.8/57</td>
</tr>
<tr>
<td></td>
<td>4.8/15</td>
<td>4.5/31.8</td>
<td>4.0/15</td>
</tr>
<tr>
<td></td>
<td>3.9/18</td>
<td>3.4/14.5</td>
<td></td>
</tr>
<tr>
<td>B.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total no. of unrelated females studied</td>
<td>59</td>
<td>65</td>
<td>48</td>
</tr>
<tr>
<td>No. of heterozygotes for RFLPs/%</td>
<td>21/35</td>
<td>47/73</td>
<td>31/48</td>
</tr>
<tr>
<td>C.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total no. of unrelated obligate carriers studied</td>
<td>27</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>No. of heterozygotes for RFLPs/%</td>
<td>9/30</td>
<td>19/82</td>
<td>14/60</td>
</tr>
</tbody>
</table>

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**Fig 1. Autoradiogram of leukocyte DNA hybridized to DX13 (left), St14 (middle), and FVIII probes (right) following digestion with BglII, TaqI, and BclI, respectively. Arrows: restriction fragment length polymorphisms (RFLPs) at each locus were investigated and were: 5.8 and 2.8 kb for DX13; 4.8, 4.5, 4.0, 3.9, 3.4 kb for St14; 0.8 and 1.1 kb for FVIII.**
HEMOPHILIA A BY DNA ANALYSIS

The criteria. Sibships (group I) and in 34 with one or more different sibships (group I) and in 34 with one or more hemophiliacs in only one sibship (group II). In the first group in which inheritance was clearly established, we investigated 29 women at risk of being carriers on the basis of genetic criteria. The BcII site revealed by the factor VIII probe was informative in 30% of cases, the TaqI system revealed by the St14 probe in 82%, and BglIII revealed by the DX13 probe in 60%. RFLP analysis allowed classification of 11 (33%) women as carriers and 18 (67%) as non-carriers of the hemophilia A gene. None of the possible carriers tested escaped identification. In obligate carriers, the ratio of factor VIII-C/vWF:Ag was in the carrier range in 16 of 19 females (84%) investigated. Definition of the carrier status by RFLP analysis agreed with the results of the ratio of conventional factor VIII-C/vWF:Ag in 12 of 13 individuals in whom both assays were carried out.

In the sporadic cases (group II), the factor VIII probe was informative in 16 of 34 families (47%), the St14 was informative probe in 20 of 30 (66%), and the DX13 probe was informative in 15 of 30 (50%). The combination of the St14 and factor VIII probes was informative in 29 of 34 families (86%). By adding the analysis of the DX13 probe, the number of informative families was increased to 32 of 34 (94%). In only two of 34 families (6%) no informative RFLPs were found. RFLP analysis allowed exclusion of the carrier status in 39 of 45 (86%) female relatives of sporadic cases. In those families in which the mother was classified as a carrier on the basis of the conventional factor VIII assay, RFLP analysis allowed us to establish the carrier status in all four investigated females at risk. The biologic assay and the length of the restriction fragment length polymorphisms (RFLPs) of these three loci (bottom). In III-5, a recombination occurred between DX13 and St14/FVIII.

RFLP analysis were concordant in 17 of 25 carriers investigated. In 7 of 12 families, in which members of three generations were available for study, the combination of the biologic assay with RFLP analysis revealed a de novo occurrence of hemophilia A mutation in the maternal grandfather. An example of this type of de novo mutation is shown in Fig 3.

Prenatal diagnosis. Prenatal diagnosis was requested by ten pregnant women from hemophilia A families (Table 4). At least 1 of the 3 polymorphisms investigated was informative in all but 1 family. In this family, the fetus was male and the pregnancy was electively terminated. Carrier status was excluded by RFLP analysis in 2 women. In the remaining 7 pregnancies, 2 fetuses were determined to be female and no further analysis was carried out. In the remaining 5, prenatal diagnosis was accomplished by RFLP analysis in 3, using the St14 probe in 2 and the DX13 probe in 1. DNA analysis predicted a normal and 2 affected fetuses, and the diagnoses were confirmed by fetal or cord blood analysis. In the remaining 2 cases with male fetuses, chorionic villous biopsy did not yield a sufficient amount of DNA for analysis. Prenatal diagnosis of an affected fetus was accomplished by fetal blood analysis in 1 case; in the other case, fetoscopy was unsuccessful and the parents decided to continue the pregnancy.

Table 2. Linkage of Factor VIII Locus to Other Markers

<table>
<thead>
<tr>
<th>Parwise Cross</th>
<th>No. of Recombinants</th>
<th>Recombination Fraction</th>
<th>Lod Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVIII × St14</td>
<td>0/36</td>
<td>0</td>
<td>4.2</td>
</tr>
<tr>
<td>FVIII × DX13</td>
<td>1/31</td>
<td>0.045</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Table 3. Hemophilia A Carrier Detection by RFLP Analysis

<table>
<thead>
<tr>
<th>Factor</th>
<th>Group I</th>
<th>Group II</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of families</td>
<td>27</td>
<td>34</td>
</tr>
<tr>
<td>No. of possible carriers investigated</td>
<td>29</td>
<td>45</td>
</tr>
<tr>
<td>Carriers' status excluded (%)</td>
<td>67</td>
<td>86</td>
</tr>
<tr>
<td>Carriers' status identified (%)</td>
<td>33</td>
<td>14</td>
</tr>
<tr>
<td>Defined by (%)</td>
<td>Bcl/FVIII</td>
<td>30</td>
</tr>
<tr>
<td>TaqI/ST14</td>
<td>82</td>
<td>50</td>
</tr>
<tr>
<td>BglIII/DX13</td>
<td>60</td>
<td>41</td>
</tr>
<tr>
<td>Bcl/FVIII-TaqI/St14</td>
<td>91</td>
<td>86</td>
</tr>
</tbody>
</table>

Fig 2. Pedigree, illustrating a crossing-over between the DX13 and the St14/FVIII loci. Bars: three chromosome haplotypes and the length of the restriction fragment length polymorphisms (RFLPs) of these three loci (bottom). In III-5, a recombination occurred between DX13 and St14/FVIII.

Fig 3. Pedigree illustrating a de novo occurrence of hemophilia A mutation. Numbers below the symbols denote the alleles of the St14 locus. Factor VIII biologic activity was measured by the FVIII/von Willebrand factor (vWF) ratio in the available females. These ratios were normal (N) in all except in II-1, in which it was decreased. Thus, the X chromosome with the 4-kb St14 allele that she passed to the affected son (III-2) already carried the hemophilia mutation. Because II-3 did not have hemophilia and II-3 is not a carrier, the mutation must have occurred in the grandfather (I-1).
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PREGNANCIES MONITORED 10
Informative 9
RFLP analysis
Noninformative 1 (Male, aborted)
Exclusion of carrier status by RFLP analysis 2
Sex determination of the fetus F 2
M 8
Diagnosis by RFLP analysis 3 (2 by TaqI-St14, 1 by BglII-DX13)
Failures 2

DISCUSSION

This study shows that a combination of three DNA probes—factor VIII, St14, and DX13—will permit detection of the carrier status in the most hemophilia A families in which at least two subjects are affected in separate sibships (group I in Table 3). Use of the factor VIII, St14, and DX13 probes alone or in various combination allowed detection of 96% of the carriers. In families with sporadic patients of hemophilia A, when informative family members were available, RFLP analysis allowed exclusion of the carrier state in 86% of female relatives (Table 3). Despite this, RFLP analysis can be used for carrier identification only when the mother of the sporadic patients has been identified as a carrier on the basis of the conventional factor VIII assay.

RFLP analysis enabled us to establish a de novo occurrence of the hemophilia A mutation in the maternal grandfather in 7 of 12 (58%) families of sporadic cases in which members of three generations were available for analysis. This indicates a high frequency of spontaneous mutation of the hemophilia A gene, in agreement with previous estimates.32-33 This study clearly indicates that carrier detection and prenatal diagnosis require testing of the mother, brother, affected and nonaffected siblings, as well as maternal grandparents and, whenever possible, the father. BclI polymorphisms within the factor VIII gene should be used primarily for carrier detection and prenatal diagnosis of hemophilia A because misdiagnosis caused by recombination between this polymorphic site and the hemophilia gene is negligible. In our experience, the intragenic BglII polymorphism was not useful since it did not increase the information obtained using the BclI polymorphism. Investigation of other polymorphisms in the factor VIII gene, particularly the recently discovered XbaI polymorphism within intron 22,34 should be a useful addition to the available markers. In families in which the polymorphic sites within the factor VIII gene are noninformative, linkage analysis with St14 and DX13 probes, closely linked to the hemophilia A locus, can be applied. In this study, the St14 locus showed no recombination with the factor VIII gene, whereas one recombinant was observed between the DX13 locus and the factor VIII/St14 loci. Previous studies have shown two crossovers between DX13 and the factor VIII gene18-22 and one between the DX13/St14 loci and the factor VIII gene.35 These results allow us to order the three loci along the X chromosome as follows: DX13/St14/factor VIII. In this study, the 95% confidence interval for probability of a recombination between the factor VIII locus and the St14 probe was 6.4% and that between the factor VIII locus and the DX13 site was 17.4%. Use of these random probes, particularly the DX13 probe thus carries a small risk of error, either in carrier detection or prenatal diagnosis. Despite this, DNA analysis for carrier detection has definite advantages as compared with the biologic assay, which is accurate in carrier identification in 70% to 95% of cases.34 Because of the risk of misdiagnosis, however, carrier detection by linkage analysis with extragenic polymorphic sites detected by random probes must be combined with the conventional factor VIII assay. Computer analysis may be used to calculate the genetic risk on the basis of pedigree information, RFLP segregation, and biological assay. Prenatal diagnosis of hemophilia A was accomplished successfully in 7 of the 10 cases investigated. Failures were caused by insufficient amount of chorionic villous DNA for analysis in two cases and uninformative RFLPs in one. From the data produced in this study, as well as in those already reported, we confidently predict that prenatal diagnosis of hemophilia A by linkage analysis with intragenic or extragenic polymorphic sites will be accomplished in ~95% of families in which a hemophilia A proband is available. According to recently published studies,34 ~30% of women at risk for being carriers of hemophilia A on genetic criteria have no living hemophilic male or father to track the mutant gene.34 In these instances, conventional factor VIII assay of fetal blood will still be required in the future, even as more polymorphic sites within the factor VIII gene and or new random probes are identified.35

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REFERENCES

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