Carrier Testing in Hemophilia B With an Immunoassay That Distinguishes a Prevalent Factor IX Dimorphism


Immunosassays with a monoclonal antibody (A-1) detect a prevalent dimorphism in plasma coagulation factor IX. The antibody was shown to react with a dimorphic segment of the normal factor IX sequence as follows. First, A-1 bound to isolated activation peptide (residues 146 through 180) prepared from activated factor IX from a normal plasma pool. Second, binding of recombinant factor IXs with A-1 or factor IX from normal individuals was strong only when they had Threonine (Thr) at position 148; factor IXs with the Alanine (Ala) allele at that position were far less reactive. Third, immunoblot reactivity of Escherichia coli fusion proteins containing known segments of the factor IX sequence restricted the epitope to residues 147 through 153. In 120 hemophilia B pedigrees, the Ala immunosassay allele frequency was 0.19 and did not differ from the Ala frequency in normal males. In 22 of 49 families, immunosassay testing was informative for classification of obligate or possible carriers. In one large family, 4 obligate carriers were heterozygous for the dimorphism and 3 of their 7 daughters were classified as carriers. In other families, when the affected member had <1 nmol/L factor IX antigen, heterozygosity for Thr/Ala alleles excluded the carrier state even when DNA studies were not informative. Strong linkage disequilibrium of Thr/Ala alleles with the common TaqI DNA polymorphism was found. Nineteen of 75 normal and hemophilic factor IX genes had the 1.3-kilobase (kb) fragment and coded for the Ala allele; the rest had the 1.8-kb fragment and coded for Thr. In selected families, the A-1 immunosassay is an inexpensive and rapid method to confirm and supplement restriction fragment length polymorphism analyses of DNA for carrier testing.

DEFICIENCY of factor IX clotting activity causes hemophilia B, a hereditary bleeding disorder with clinical manifestations indistinguishable from those seen with factor VIII deficiency. The molecular defects leading to hemophilia B are heterogeneous. Single amino acid substitutions, a donor splice junction defect, and gene deletions of various size have been described.

In kindreds with readily measurable dysfunctional plasma factor IX, the expression of excess antigen as compared with clotting activity has been used to detect carriers. In some kindreds, the abnormal factor IX expressed differs in mol wt from native factor IX and can be distinguished from normal factor IX on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), but carrier detection using this technique has not been described.

Accuracy of carrier testing based on expression of the abnormal gene is limited by random inactivation of X chromosomes in female hepatic cells and by the wide distribution of factor IX antigen and activity among normal subjects. In contrast, the accuracy of family studies for carrier testing using intragenic restriction fragment length polymorphisms (RFLPs) is limited only by technical or sample errors or by unlikely recombination in the family tested. Quantitative cDNA hybridization studies may also detect gene deletions in potential carriers. RFLP analyses, although well established, are time-consuming and expensive.

In 1985, McGraw et al described a prevalent DNA and protein polymorphism at amino acid residue 148 in normal and hemophilic factor IX. They predicted that the threonine (Thr) allele was more prevalent than alanine (Ala) in the general population. Recently, carrier testing by oligonucleotide probes specific for the Thr or Ala codons has been described. The codon for the Ala allele was present at a frequency of 0.33 (95% confidence interval, 0.21 to 0.45) in the normal population. Two independent reports have shown that monoclonal antibody immunosassays can detect a dimorphism in plasma factor IX. The A-1 antibody distinguishes factor IX alleles with a frequency of 0.25 for the less frequent form (95% confidence interval of 0.14 to 0.36). The current study demonstrates the specificity of the A-1 monoclonal antibody for an epitope near the amino terminus of the activation peptide of factor IX. This epitope includes residue 148, which is dimorphic. The A-1 antibody binds well to factor IX with Thr at residue 148 and weakly to factor IX with Ala at that site. Thus, the dimorphism is the basis for immunoassay reactivity. Heterozygous women show intermediate immunoassay reactivity and, in families in which abnormal factor IX is expressed, the assay can be used as a rapid initial screen to aid in hemophilia B carrier testing.

MATERIALS AND METHODS

Materials. Sephadex G-100, diethylaminoethyl (DEAE)-Sephadex CL-6B, activated CH-Sepharose 4B, and electrophoresis mol-wt standards were from Pharmacia, Piscataway, NJ. Acetonitrile and benzamidine were from Aldrich Chemical, Milwaukee, WI. Affigel-10, acrylamide, bis-acrylamide, ammonium persulfate, and N, N', N'-tetramethylethylenediamine were from BioRad, Rich-

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mmond, CA. Low-mol-wt electrophoresis standards were from Pierce, Rockford, IL. TagI was from New England Biolabs, Beverly, MA or BRL, Bethesda, MD. Peroxidase was from Boehringer Mannheim Biochemicals, Indianapolis, and α-phenylenediamine was from Sigma Chemical, St Louis.

**Factor IX preparations.** Factor IX was partially purified from plasma pooled from 20 to 30 patients undergoing therapeutic plasmapheresis.\(^14\) In the final purification step, 5 to 20 mg factor IX in 0.05 mol/L NaCl/0.05 mol/L Tris-HCl (pH 7.2) to 60 mmol/L benzamidine HCl/20 mmol/L MgCl\(_2\) was passed over a 1 x 8-cm column of A-7 antibody (anti-light chain of factor IX),\(^12\) coupled to Affigel-10 at a concentration of antibody 3 mg/mL gel. The column was washed with three volumes of 1.0 mol/L NaCl/0.02 mol/L Tris-HCl (pH 7.2)/20 mmol/L MgCl\(_2\)/1 mmol/L benzamidine HCl and then eluted with 20 mmol/L EDTA in 0.15 mol/L NaCl/0.02 mol/L Tris-HCl (pH 7.2)/1 mmol/L benzamidine HCl. Factor IX from individual male patients undergoing plasmapheresis was purified by batch adsorption and elution from an ion-exchange matrix, as previously described,\(^{21}\) with the modification that DEAE-Sepharose was used instead of DEAE-Sephadex. The DEAE-Sepharose eluate was precipitated with 65% saturated ammonium sulfate and then dissolved in 0.15 mol/L NaCl/0.02 mol/L Tris-HCl (pH 7.2)/10 mmol/L benzamidine HCl/20 mmol/L MgCl\(_2\) before isolation of factor IX by monocolonal antibody immunoaffinity chromatography. Factor IX preparations were homogenous by SDS-PAGE.

Tissue culture supernatants with recombinant factor IX were provided by Dr George Brownlee, Cambridge, England,\(^{24}\) Dr Sharon Busbee, Zymogenetics, Seattle,\(^{11}\) Dr Charles Shoemaker, Genetics Institute, Cambridge, MA,\(^{29}\) and Dr Henri De LaSalle, Transgene S.A., Strasbourg, France.\(^{25}\) Immunoassays were done on tissue culture supernatants from Dr Brownlee and Dr De LaSalle that had been previously lyophilized. Otherwise, samples had been stored at -70°C.

**Factor IX activation and activation peptide isolation.** Factor IX (0.5 to 1.0 mg/mL), prepared from pooled plasma or individual males was activated for 4 hours with solid-phase crude factor Xla\(^{26}\) in 0.1 mol/L NaCl/0.05 mol/L Tris-HCR (pH 8)/10 mmol/L CaCl\(_2\). Factor Xla was coupled to activated CH-Sepharose at a concentration of 5 to 10 mg/mL gel. The volume of factor Xla-Sepharose added was 5% of the factor IX solution. After addition of EDTA to a final concentration of 20 mmol/L, solid-phase Xla was removed by centrifugation and the supernatant was dialyzed against 0.1 mol/L NaHCO\(_3\) and lyophilized. In one preparation of factor IX, the activation peptide was purified from monocolonal antibody immunoaffinity chromatography of factor IX by on-column affinity chromatography with 0.1% benzamidine HC\(_1\)/20 mmol/L MgCl\(_2\) was passed over a 1 x 8-cm column of Laemmli.\(^{27}\) The DNA was isolated,\(^{27}\) and the single-strand phage M13 mpl8 for dideoxy sequencing using nonradioactive A-l antibody followed by 125I-protein A (ICN, Cleveland).

As previously described,\(^{24}\) a Xgt11 expression library was constructed containing random, small DNase 1 fragments of the coding region of a human factor IX cDNA. The recombinant phage were screened for immunoreactivity, using A-1 monoclonal antibody and 125I-protein A or 125I-A1 prepared by chloramine-T technique.\(^{27}\) Ten-milliliter aliquots of the purified recombinant phage were grown from the pure phage stocks, the DNA was isolated,\(^{13}\) and the EcoRI fragment containing factor IX cDNA was ligated directly into the single-strand phage M13 mp18\(^{26}\) for dideoxy sequencing using 35-S-dATP.\(^{37}\)

**Immunoblotting of factors IX and IXa with A-1.** Two- to 5-μg samples of factor IX or factor IXa were applied to lanes of Laemmli gels and electrophoresed prior to transfer to nitrocellulose. After nonspecific binding was blocked by soaking the nitrocellulose in 5% nonfat dry milk in 0.15 mol/L NaCl/0.02 mol/L Tris-HCl (pH 7.2) with 0.2% sodium azide, radiiodinated monocolonal antibodies with a specific activity of 2 μCi/μg were diluted to 10^6 cpn/mL in the same solution for reaction with nitrocellulose for 2 hours at room temperature. Nitrocellulose sheets were washed in buffer without 5% milk solids three times and then exposed overnight for radiography.

**Studies on human subjects.** Plasma from patients undergoing therapeutic plasmapheresis was obtained from United Blood Services of Albuquerque. Informed consent for blood samples was obtained as approved by the University of Washington's Human Subjects Review Committee. One hundred twenty hemophilia B...
pedigrees with >2 nmol/L factor IX antigen concentration were tested by immunoassay, as were females from 49 families. EDTA plasma samples and DNA from 39 unrelated normal males from families with hereditary disorders other than hemophilia B were tested for immunoassay alleles and TaqI RFLPs using a factor IX cDNA probe. TaqI RFLPs and immunoassays were also compared for affected members in 28 hemophilia B kindreds.

RESULTS

A-1 reactivity with factor IX activation peptide. Factor IX activation peptide was purified from factor IX obtained from pooled plasma and from 2- to 3-L amounts of plasma from male patients undergoing therapeutic plasmapheresis. The late eluting peak from gel filtration of factor IXa preparations from an individual male plasma and from pooled plasma were resolved into three peaks (Fig 1A) when analyzed on reverse-phase HPLC. The first two peaks (I and II, Fig 1A) did not stain with Coomassie blue but were readily stained with periodic acid-Schiff (PAS) reagent and showed identical migration on SDS-PAGE (Fig 1B, gel 1). The third peak (III, Fig 1A) did not stain with PAS but could be stained with Coomassie blue (Fig 1B, gels 2 and 3). PAS-positive peaks resolved by HPLC did not migrate at the expected position for factor IX activation peptide on SDS-PAGE using either Laemmli gels (pH 8.8) or Weber Osborn gels (pH 7.0). When the percentage of acrylamide in gels was 12.5%, the apparent migration of the glycopeptide was 30,000 mol wt, whereas on 15% gels, the apparent migration was 25,000 mol wt. The gel migration and staining properties of activation peptide differ from those previously described.

Samples obtained by HPLC were analyzed by N-terminal sequence analysis and for amino acid content. PAS-positive peaks (I and II) obtained on HPLC of activation peptide from pooled plasma were identical in N-terminal sequence and amino acid content. The first eight residues for both peaks gave the sequence Ala-Glu-(Thr/Ala)-Val-Phe-Pro-Asp-Val, corresponding to residues 146 through 153 in factor IX.

Observed and (expected) values for amino acid content of peak II were as follows: Asp 7.3 (7), Thr 5.6 (6), Ser 2.6 (3), Glue 5.1 (5), Ala 2.2 (2), Val 3.0 (3), Ile 1.9 (2), Leu 1.1 (1), Tyr 0.8 (1), Phe 3.7 (3), and Arg 1.1 (1). Proline was not detected with orthophthalaldehyde in the method used for amino acid analysis. The expected values given are for the more prevalent sequence containing Thr rather than Ala and Thr and Ser content is corrected for loss on hydrolysis. The glycopeptide from either HPLC peaks I or II was able to inhibit the binding of the A-1 antibody to factor IX immobilized on microtiter wells (Fig 2). These peptides did not inhibit the binding of other monoclonal antibodies to immobilized factor IX (data not shown).

The peak with the greatest retention time on reverse-phase HPLC (peak III, Fig 1A; Fig 1B, gels 2 and 3) did not inhibit the binding of the A-1 antibody to solid-phase factor IX and did not stain with PAS. Peptides in this peak were a minor component of the activation peptide preparation from gel filtration of IXa, since their N-terminal residues were not detected in N-terminal amino acid sequence analysis. These peptides were probably cleaved from the heavy chain of factor IXa by contaminating proteases in the crude celite eluant (amino acid sequence data not shown).

A-1 reaction with activation peptide was further tested with Western blotting. Native factor IX and an activation intermediate of factor IX (heavy chain of IXa and factor IXa activation peptide) were recognized by the A-1 antibody, but the heavy chain of factor IXa was not (Fig 3). Antibody binding was not seen in the position expected for the activation peptide itself, although tritium label could be detected on nitrocellulose in the expected migration position for activation peptide after SDS-PAGE and electrophoretic
transfer of tritiated factor IXa (data not shown). When iodinated-staphylococcal protein A and nonradioactive A-1 had been used for radioautography in previous experiments, there was greater background binding, erroneously interpreted as A-1 binding to the heavy chain of factor IXa.

Specificity of A-1 for the Thr allele. Amino acid sequence analysis on isolated activation peptide prepared from pooled plasma showed both Thr and Ala at position 3. The ratio of Thr to Ala of position 3 was calculated as 3.8 to 1 based on a repetitive yield of 21% of the expected Ala yield at turn 3 given yields of Val at turns 4 and 8. This result is similar to the predicted prevalence of 3 : 1 of Thr over Ala in pooled plasma from which the factor IX was derived. Partially sequenced factor IX activation peptide from each of three males with the more prevalent immunoassay allele (with strong A-1 binding) had only Thr at position 148, and one sample with the less frequent allele in the immunoassay (weak A-1 binding) had only Ala at this position (Table 1).

Factor IX produced by recombinant DNA technology was tested in a two-antibody immunoassay system using the A-1 antibody and the antigenic determinant to the heavy and light chains of factor IXa was found. Studies with fusion proteins. The region of factor IX recognized by A-1 was also defined by its reaction with specific fragments of human factor IX expressed in E coli under control of the T7 gene 10 promoter and reaction with a series of overlapping random fragments of human factor IX cDNA expressed in λ gt11 as fusion proteins to β-galactosidase. The specific fragments made under the control of the T7 promoter included amino acid residues 3 through 180, 1 through 49, 50 through 111, 112 through 142, 116 through 180, 147 through 180, 180 through 265, 266 through 324, 335 through 415 and 3 through 415 (fragments marked with an asterisk reacted with A-1). Reactivity with the specific fragments limit the antigenic determinant to the activation peptide. To define the epitope further, the random fragment library in λ gt11 was screened with A-1, positive plaques were purified, and the DNA insert was sequenced. Approximately 1 in 1,000 clones was reactive with A-1.

One λ gt11 insert was sequenced and found to extend from base 352 to base 460 of the zymogen cDNA coding sequence (17,767 to 17,797 and 20,363 to 20,480 in the numbering of Yoshitaki). This insert codes for a peptide extending from Ala 118 to Val 153. A second nucleotide cloned was sequenced and was identical except for being one base shorter. The overlap between these fragments and a reactive fragment of the specific T7 gene 10 promoter system is amino acids 147 through 153 of the activation peptide, including residue 148 with the polymorphism (Fig 4).
cDNA expressed had the ACT codon at bases 20,422 through 20,424 for Thr, accounting for reactivity of its expressed fragments with A-1.14

**Human subjects.** Plasma samples from 120 kindreds with hemophilia B and >2 nmol/L factor IX antigen concentration (>2.0 U/dL or >2% of normal) were tested by immunoassay to determine the number of individuals expressing Thr or Ala. The frequency of the Ala allele was 0.19 (95% confidence interval, 0.11 to 0.28). This did not differ from the frequency of 0.25 in normal males (95% confidence interval, 0.14 to 0.36). In 12 kindred in which more than one affected individual was available for testing, the immunoassay Thr/Ala classification was consistent for the abnormal gene product within each family.

The association of the *TaqI* 1.8-kb fragment with the Thr allele and the 1.3-kb allele with the Ala allele by immunoassay result was 100% in affected members from 28 tested kindreds with hemophilia B. The association of the two alleles with these *TaqI* fragments in 39 unrelated normal males was also 100% (Table 2), indicating strong linkage disequilibrium of the DNA and protein polymorphisms.

Of the 114 females from 48 additional hemophilia B families, 53 were from 27 families in which immunoassays were not informative for carrier detection. In the other 21 families, immunoassays in 40 of 61 were informative. These include 27 women who were Thr/Ala heterozygotes and nine women with a homozygous phenotype different from the immunoassay allele expressed in the hemophilic gene product. In four families, the sons of obligate carriers had <1 nmol/L factor IX antigen. The carrier state could be excluded in four women in three of these families because they expressed nearly equal amounts of the Thr/Ala alleles and had normal factor IX antigen levels. In two of these families, RFLP analyses of DNA were not informative (data not shown).

Twenty-four samples from the large pedigree shown in Fig 5 and Table 3 were tested by immunoassay for the Thr and Ala alleles. Four obligate carriers (III-5, III-6, III-7, and III-8) had intermediate reactivity to A-1, indicating that they were heterozygous for the Thr and Ala alleles. The hemophilic gene was associated with the Thr allele in all five affected family members tested (II-1, III-10, IV-4, IV-8, IV-12). All four nonaffected males who were children of the four heterozygous carriers had the Ala allele (IV-6, IV-7, IV-10, IV-11). The carrier status of seven possible carriers could be predicted in generation IV by the immunoassay result. Four tested as noncarriers (IV-5, IV-9, IV-16, IV-17), and three were predicted to be carriers (IV-13, IV-14, IV-15) (Table 3, Fig 5).

Carrier testing was confirmed by restriction fragment polymorphisms. Leukocyte DNA was obtained from two patients (IV-8 and IV-12) and five other family members (III-5, III-7, and III-7a and IV-13, IV-14). DNA was digested with *TaqI* and hybridized to a cDNA probe. The

**Table 1. Factor IX Antigen Concentration (nmol/L) by Immunoassay**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Immunoassay A-1, A-7*</th>
<th>Immunoassay A-5, A-7*</th>
<th>Amino Acid at Residue 148</th>
<th>Codon</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Pooled plasma</td>
<td>67</td>
<td>89</td>
<td>Thr/Ala (4:1)</td>
<td>—</td>
</tr>
<tr>
<td>2. Male donor A</td>
<td>127</td>
<td>131</td>
<td>Thr</td>
<td>—</td>
</tr>
<tr>
<td>3. Male donor B</td>
<td>91</td>
<td>86</td>
<td>Thr</td>
<td>—</td>
</tr>
<tr>
<td>4. Male donor C</td>
<td>93</td>
<td>83</td>
<td>Thr</td>
<td>—</td>
</tr>
<tr>
<td>5. Male donor D</td>
<td>9</td>
<td>90</td>
<td>Ala</td>
<td>—</td>
</tr>
<tr>
<td>6. rDNA IX22</td>
<td>&lt;0.09</td>
<td>1.1</td>
<td>—</td>
<td>GCT</td>
</tr>
<tr>
<td>7. rDNA IX24</td>
<td>&lt;0.45</td>
<td>9.1</td>
<td>—</td>
<td>GCT</td>
</tr>
<tr>
<td>8. rDNA IX23</td>
<td>&lt;0.18</td>
<td>8.6</td>
<td>—</td>
<td>GCT</td>
</tr>
<tr>
<td>9. rDNA IX26</td>
<td>7.4</td>
<td>7.7</td>
<td>—</td>
<td>ACT</td>
</tr>
</tbody>
</table>

Samples of recombinant factor IX, pooled normal plasma, or plasma from male donors undergoing therapeutic plasmapheresis were tested in an immunoassay system in which either the A-1 (anti-activation peptide) or A-5 (anti-factor IXa heavy chain) was used as the solid-phase antibody in a two-antibody immunoradiometric assay (described in text). After incubation of diluted plasma or tissue culture supernatant samples in the wells, bound antibody was detected with radiiodinated A-7 antibody (A-7*). The radio iodinated antibody in the two antibody assays is indicated by an asterisk. Control media with 10% fetal calf serum had <0.09 nmol/L factor IX antigen content. The factor IX concentration in pooled plasma is assumed to be 89 nmol/L. Plasma levels of factor IX measured with the A-1, A-7* immunoassay system were determined by comparison with standard curve generated by dilution of plasma factor IX from a single male with the Thr allele. The codon numbering is that of Yoshitaki et al.12 Reference numbers are given for the source of recombinant DNA factor IX samples.

![Fig 4. Diagram of A-1 epitope. Factor IX regions encoded by two immunoreactive clones: (A) a λ gt11 insert; (B) a fragment from the T7 expression system. The overlap region (shaded) must include part of the epitope recognized by A-1.](www.bloodjournal.org)
two affected males and the one father (III-7a) examined had the more frequent 1.8-kb band whereas the obligate carriers (III-5, III-7) were heterozygous with 1.8-kb and 1.3-kb bands. Both potential carriers (IV-13 and IV-14) had the 1.8-kb fragment alone, indicating that they had inherited their mother’s hemophilic factor IX gene, confirming results of the immunoassay studies.

**Table 3. Heterozygote Detection by Monoclonal Antibody Specific for Thr-148 Allele**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Status</th>
<th>IX:C</th>
<th>IX:Ag (non, A-i)</th>
<th>IX:Ag (A-i)</th>
<th>X-linked Genotype (Assigned)</th>
<th>TaqI Genotype (kb)</th>
<th>Polymorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td>II-1</td>
<td>M</td>
<td>Hemophilic</td>
<td>26</td>
<td>62</td>
<td>110</td>
<td>Thr-H</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>II-2</td>
<td>M</td>
<td>Hemophilic</td>
<td>25</td>
<td>—</td>
<td>—</td>
<td>(Thr-H)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>III-3</td>
<td>F</td>
<td>Obligate</td>
<td>64</td>
<td>72</td>
<td>95</td>
<td>Thr-H/Thr-N</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>III-5</td>
<td>F</td>
<td>Obligate</td>
<td>60</td>
<td>49</td>
<td>18</td>
<td>Thr-H/Ala-N</td>
<td>1.8/1.3</td>
<td>—</td>
</tr>
<tr>
<td>III-6a</td>
<td>M</td>
<td>Normal</td>
<td>108</td>
<td>121</td>
<td>9</td>
<td>Ala-N</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>III-6b</td>
<td>F</td>
<td>Obligate</td>
<td>39</td>
<td>42</td>
<td>22</td>
<td>Thr-H/Ala-N</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>III-7</td>
<td>F</td>
<td>Obligate</td>
<td>—</td>
<td>70</td>
<td>16</td>
<td>Thr-H/Ala-N</td>
<td>1.8/1.3</td>
<td>—</td>
</tr>
<tr>
<td>II-7</td>
<td>F</td>
<td>Normal</td>
<td>120</td>
<td>160</td>
<td>190</td>
<td>Thr-N</td>
<td>1.8</td>
<td>—</td>
</tr>
<tr>
<td>III-8</td>
<td>F</td>
<td>Obligate</td>
<td>72</td>
<td>90</td>
<td>16</td>
<td>Thr-H/Ala-N</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>III-9</td>
<td>M</td>
<td>Normal</td>
<td>100</td>
<td>118</td>
<td>155</td>
<td>Thr-N</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>III-10</td>
<td>M</td>
<td>Normal</td>
<td>20</td>
<td>29</td>
<td>74</td>
<td>Thr-H</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>IV-2</td>
<td>M</td>
<td>Normal</td>
<td>76</td>
<td>76</td>
<td>100</td>
<td>Thr-N</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>IV-3</td>
<td>M</td>
<td>Hemophilic</td>
<td>15</td>
<td>27</td>
<td>44</td>
<td>Thr-H</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>IV-5</td>
<td>F</td>
<td>Possible</td>
<td>78</td>
<td>110</td>
<td>2</td>
<td>Ala-N/Ala-N</td>
<td>—</td>
<td>—</td>
</tr>
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<td>IV-6</td>
<td>M</td>
<td>Normal</td>
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<td>92</td>
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<td>Ala-N</td>
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<td>—</td>
</tr>
<tr>
<td>IV-7</td>
<td>M</td>
<td>Normal</td>
<td>78</td>
<td>74</td>
<td>1</td>
<td>Ala-N</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>IV-8</td>
<td>M</td>
<td>Hemophilic</td>
<td>20</td>
<td>27</td>
<td>40</td>
<td>Thr-H</td>
<td>1.8</td>
<td>—</td>
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<tr>
<td>IV-9</td>
<td>F</td>
<td>Possible</td>
<td>74</td>
<td>85</td>
<td>1</td>
<td>Ala-N/Ala-N</td>
<td>—</td>
<td>—</td>
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<tr>
<td>IV-10</td>
<td>M</td>
<td>Normal</td>
<td>80</td>
<td>83</td>
<td>1</td>
<td>Ala-N</td>
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<td>IV-11</td>
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</tr>
<tr>
<td>IV-12</td>
<td>M</td>
<td>Hemophilic</td>
<td>44</td>
<td>44</td>
<td>55</td>
<td>Thr-H</td>
<td>1.8</td>
<td>—</td>
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<tr>
<td>IV-13</td>
<td>F</td>
<td>Possible</td>
<td>—</td>
<td>69</td>
<td>93</td>
<td>Thr-H/Thr-N</td>
<td>1.8/1.8</td>
<td>—</td>
</tr>
<tr>
<td>IV-14</td>
<td>F</td>
<td>Possible</td>
<td>42</td>
<td>42</td>
<td>64</td>
<td>Thr-H/Thr-N</td>
<td>1.8/1.8</td>
<td>—</td>
</tr>
<tr>
<td>IV-15</td>
<td>F</td>
<td>Possible</td>
<td>45</td>
<td>61</td>
<td>72</td>
<td>Thr-H/Thr-N</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>IV-16</td>
<td>F</td>
<td>Possible</td>
<td>80</td>
<td>80</td>
<td>33</td>
<td>Thr-N/Ala-N</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>IV-17</td>
<td>F</td>
<td>Possible</td>
<td>80</td>
<td>111*</td>
<td>84</td>
<td>Thr-N/Ala-N</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Factor IX clotting activity (IX:C), antigen levels (IX:Ag) by routine (non, A-i) and specific (A-i monoclonal) and TaqI polymorphic patterns are shown for members of a large pedigree with mild hemophilia B. Clotting and antigen levels are expressed in units per deciliter (U/dL), where normal pooled plasma is assumed to have 100 U/dL. N and H designations for Thr and Ala alleles are for normal and hemophilic genes, respectively. Assay methods for A-1 and non-A-1 assays are explained in the text. All samples were compared to a standard pooled plasma except IV-17.

The female’s plasma gave comparable levels of IX:Ag by the two assays. It is probable that she is heterozygous for the Ala allele. Comparison of IX:Ag with a standard plasma from a single male with the Thr allele better defines the heterozygous state in this family member.
The carrier state for members of selected families. Analysis of DNA RFLPs is well established for carrier testing, but disadvantages are the time required for isolation and digestion of DNA, the expense of restriction enzymes, and the complexity of analysis of RFLPs when full-length cDNA is used as a probe.

The immunoassay system is useful as a rapid test that provides a valuable reference for the results of DNA analysis in families in which measurable abnormal factor IX is expressed. In addition, linkage data indicate that the heterozygosity for Thr/Ala will predict the utility of DNA analysis when heterozygosity for TaqI restriction sites is required. Thus, carriers heterozygous for Thr/Ala are likely to be heterozygous for TaqI, and prenatal diagnosis by TaqI RFLP analysis should be possible.

The immunoassay may not be useful in some families in which the abnormal gene product is not expressed in sufficient amounts for Thr/Ala classification by immunoassay. In addition, since carrier detection by immunoassay depends on expression of both X chromosome gene products in females, the accuracy of detection will be limited by random inactivation of the X chromosome in hepatocytes, as occurred in borderline values for classification of potential carrier IV-17 in the large family presented. In some families in which the abnormal factor IX gene product is not expressed or present at levels <1 nmol/L, however, the finding of both Thr/Ala alleles expressed in potential carriers can exclude the carrier state when RFLP analyses are not informative.

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REFERENCES

14. McGraw RA, Davis LM, Noyes CM, Lundblad RL, Roberts HR: A further difference between the Swedish antibody and A-1 is that the 9.9 antibody's reactivity with factor IX samples expressing the less frequent allele was 1,000-fold less than polyclonal antibodies or other monoclonal antibodies tested. Although the A-1 antibody has reduced reactivity with factor IX samples expressing Ala, in some cases the apparent antigen is as high as one-fifth of that seen with polyclonal and other monoclonal antibodies. The localization of the epitope to which the Swedish antibody binds and the potential overlap with the sequence to which A-1 binds remains to be established.

The sequence data on the activation peptide confirm the previous report on the activation peptide dimorphism. Three of four individuals showed Thr and their plasma factor IX reacted strongly with A-1, whereas one individual whose activation peptide contained Ala had reduced reactivity in factor IX immunoassays. The possibility that there may be an additional, linked polymorphism in the activation peptide is unlikely since the cDNA sequence of factor IX has been determined by five groups, and the only difference in these is the codon specifying Thr or Ala at position 148. In addition, recombinant factor IX proteins showed A-1 reactivity as predicted from their known DNA sequence.

The nature of the structural difference between the Thr peptide and the Ala peptide is not known, but there is no apparent difference in clotting activity between the two variants. Because the analysis of A-1 reactivity of hemophilic plasmas could be altered by mutations in regions adjacent to or including the activation peptide, it will be important to test the immunoassay classification for Thr and Ala alleles in hemophilia B samples in which amino acid substitutes are known to be adjacent to the amino terminal region of the activation peptide. Verification of the A-1 classification for hemophilic factor IX could also be obtained by using oligonucleotide probes specific for the DNA polymorphism coding for Thr or Ala in hemophilic DNA.


37. Biggin MD, Gibson TJ, Hong GF: Buffer gradient gels and 35S label as an aid to rapid DNA sequence determination. Proc Natl Acad Sci USA 80:3963, 1983


Carrier testing in hemophilia B with an immunoassay that distinguishes a prevalent factor IX dimorphism

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