

CONCISE REPORT

Hemophilia B With Inhibitor: Molecular Analysis of the Subtotal Deletion of the Factor IX Gene

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The structure of factor IX gene was analyzed in a hemophilia B patient with inhibitor. Genomic DNA, digested with a variety of restriction endonucleases, was hybridized with the cDNA and various genomic factor IX probes. A large subtotal deletion of the gene was observed. The borders of the deletion span from a ~125 nucleotide region within the

last exon to an unknown domain at least 7.5 kb upstream from the first exon: it thus involves ~33 kb of the factor IX locus. The abnormal gene was inherited by the daughter of the propositus, who showed both the normal and the deleted allele.

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FACTOR IX is a serine protease involved in the blood-clotting process.¹ Deficient levels or functional abnormality of this glycoprotein cause hemophilia B, a bleeding disorder characterized by both clinical and biochemical heterogeneity.² In 1% of the cases the therapy is complicated by the appearance of an inhibitor, specifically directed against factor IX.³ Although previous studies described a heterogeneous series of gene deletions in five out of six cases with inhibitor,^{4,5} a detailed molecular mapping of these deletions, and particularly their exact borders, has not been described as yet.

We report a patient with severe hemophilia B, who developed an inhibitor to factor IX after substitution therapy. A large deletion has been finely mapped in the factor IX locus, starting within the last coding exon and extending for >33 kb upstream.

MATERIALS AND METHODS

The patient is a 47-year-old man from southern Italy with severe factor IX deficiency, but with a history of mild bleeding. An affected brother died from post-traumatic cerebral hemorrhage. From the time of diagnosis, he was occasionally treated with fresh-frozen plasma. In 1980 he developed frequent bleeding from a duodenal ulcer and thereupon received prothrombin complex concentrates. During this period, a high-titer inhibitor (25 Bethesda U/mL) was found.

Peripheral blood samples from normal subjects and the patient were collected into 1/10 vol of 3.2% trisodium citrate. Factor IX:C levels were determined on platelet-poor plasma by the one-stage assay,⁶ and factor IX:antigen by Laurell immunoelectrophoresis.⁷ Quantitative assay of human factor IX inhibitor was performed according to the Bethesda method⁸ modified as previously described.⁹

Blood cells, washed with phosphate-buffered saline (PBS), were stored at -20 °C until used. High molecular weight DNA, obtained from peripheral leukocytes by standard techniques,¹⁰ was digested with Eco RI, Hind III, Bgl II, Bam HI, Taq I, Hinf I, and Msp I

restriction endonucleases (4 U/μg of DNA), according to the supplier's specifications (Biotech Inc, Boehringer, Mannheim, West Germany). Restricted DNA was electrophoresed on 0.8%, 1.5% and 2% agarose (Bio-Rad Laboratories, Richmond, Calif) gels, transferred onto nitrocellulose filters, and hybridized to 2 × 10⁷ cpm of ³²P-labeled DNA probe (specific activity, 3-9 × 10⁸ dpm/μg), as previously described.¹¹ The five subgenomic probes (II, III, VII, VIII, XI) and the cDNA fragment (probe V)^{4,12} were kindly provided by Dr G.G. Brownlee, Oxford.

RESULTS

Clinical results in this hemophiliac revealed that factor IX:C activity was <1 U/dL and that factor IX antigen levels were undetectable. He developed a specific antifactor IX antibody after substitution therapy: the inhibitor titer ranged between 1 and 25 Bethesda U/mL. The daughter of the patient, genetically defined as an obligatory carrier, has low levels of factor IX:C (ie, 30 U/dL).

Genomic DNA from the patient's peripheral blood white cells, digested with different restriction endonucleases and hybridized to a factor IX cDNA probe, showed only one band of abnormal size (Fig 1). The strong hybridization signal suggested that the band comprised the DNA region

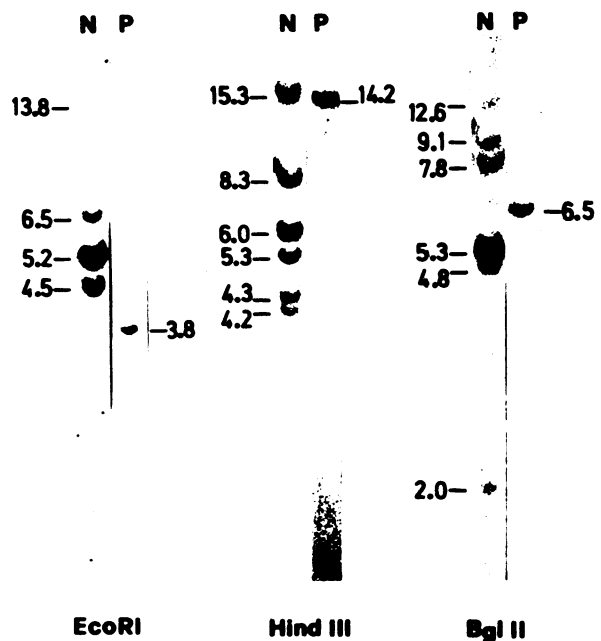


Fig 1. DNA restriction pattern of a normal subject (N) and the hemophiliac patient (P) after hybridization with the cDNA probe.

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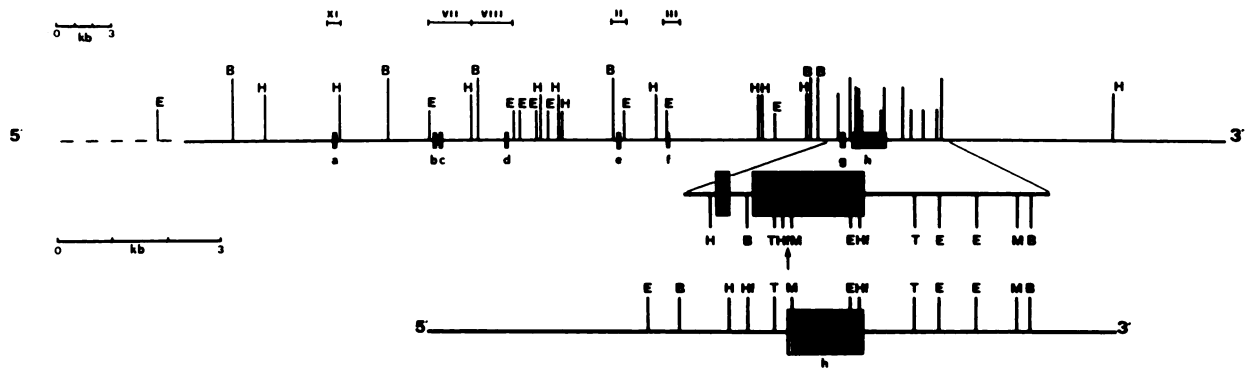


Fig 2. Top: normal factor IX gene structure and partial restriction map, including Eco RI (E), Hind III (H), Bgl II (B), Taq I (T), Hinf I (Hf), and Msp I (M) enzymes: an expanded portion of the gene is also indicated below. Bottom: the residual part of factor IX gene in the hemophilic patient. The arrow and the dashed line indicate the 3' and 5' borders of the deletion respectively. The subgenomic probes employed here are indicated by II, III, VII, VIII, and XI.

related to exon "h" (Fig 3), which represents ~1.2 out of ~2.0 kb in the employed cDNA probe. A series of genomic probes (II, III, VII, VIII, XI) were thereafter employed (Fig 2): no hybridization bands were observed whatever the enzyme and/or the genomic probe (data not shown).

The combined studies performed with genomic and cDNA probes allowed us to localize the borders of the deleted region. Using a variety of restriction endonucleases for the 3' region of the gene (Fig 2) and the cDNA probe, we observed abnormal bands up to the first Hinf I site within exon "h." On the contrary, the Msp I band related to this region was of normal size (Fig 3). Comparative evaluation of the length of the normal Eco RI, Hind III, and Bgl II fragments in the 5' region, as related to that of the abnormal bands after hybridization with the cDNA probe, allowed us to map the 5' border of the deletion. This is situated at least 7.5 kb 5' to the

first exon ("a" in Fig 2), upstream from the Bgl II site. The deletion involves at least 41 kb, 33 of which span through the factor IX locus. The abnormal gene was inherited by the daughter of the propositus, who obviously also showed the normal allele (Fig 3).

DISCUSSION

The development of a circulating inhibitor is a serious complication of hemophilias. The appearance of an inhibitor implies a severe prognosis: replacement therapy is then generally restricted to patients with life-threatening hemorrhages in an attempt to maintain the antibody at the lowest possible titer. Hemophilia B with inhibitor(s) is often associated with a factor IX gene deletion, which was observed in five out of six cases reported so far.^{4,5} The extension of the deletion is apparently heterogeneous and may involve different regions of the gene.^{4,5}

In our case, a deletion of 33 out of a total of ~34 kb of the factor IX locus¹² was observed. This is the first case in which the borders of the deletion have been mapped. Results obtained with the cDNA and different genomic probes showed that the deletion spans through the structural gene from at least 7.5 kb upstream from the first exon up to the last exon, which is partially retained. The 3' break-point in the normal locus has been mapped within a ~125 nucleotide region, ie, between a Hinf I and a Msp I site within the translated portion of the last exon "h".

The abnormal bands observed in the genomic DNA, digested with different restriction enzymes and hybridized to the cDNA probe, can be used as a marker for carrier detection and prenatal diagnosis. This approach has been successfully used for predictive diagnosis of severe α - and $\delta\beta$ -thalassemia,¹³ which are generally due to large gene deletions in the corresponding globin gene domain.¹⁴

Hemophilia B without inhibitor is characterized by the absence of gross deletions or rearrangements in the factor IX gene.^{4,15} Additionally, a Taq I restriction fragment-length polymorphism does not show a linkage disequilibrium in its clinical and biochemical variants¹⁵: this indicates that the underlying molecular abnormalities are heterogeneous. In the light of the molecular studies carried out on thalassemia syndromes,¹⁴ it may be predicted that these abnormalities

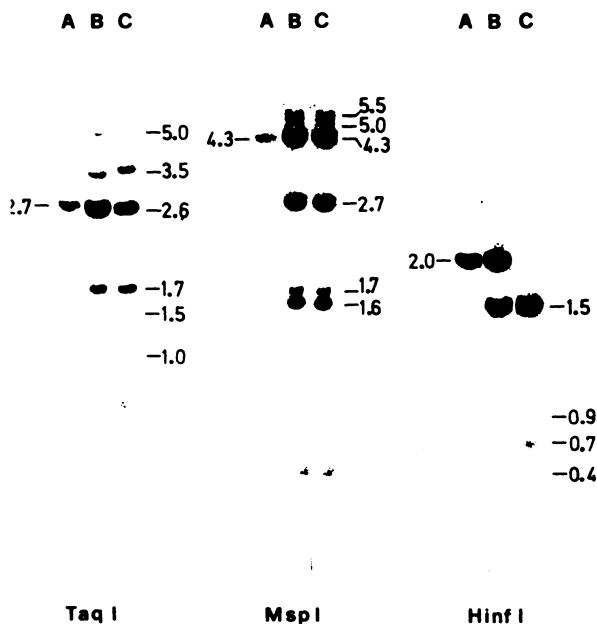


Fig 3. DNA restriction fragments pattern of the hemophilic patient (A), his daughter (B), and a normal subject (C) after hybridization with the cDNA probe.

include a variety of point mutations in critical DNA sequences, possibly leading to altered RNA splicing (CRM-negative forms) and/or abnormal protein molecules with reduced or absent clotting function (CRM-positive forms). Along these lines, it shall be interesting to verify whether hemophilia B with inhibitor but without a deletion⁴ is caused by a nonsense mutation, leading to premature termination of

RNA translation, as observed in most cases of β^0 -thalassaemia.¹⁶

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