CONCISE REPORT

The Putative Factor IX Gene Promoter in Hemophilia B Leyden

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Hemophilia B Leyden is characterized by low levels of factor IX antigen and activity before the age of 15, whereas after puberty factor IX levels rise at a rate of about 5% per year. A single base substitution (A → T) at position −20 was identified in the putative promoter of the gene cloned from a patient with hemophilia B Leyden. This nucleotide change was confirmed in a second patient from the same pedigree and was also found in a patient from a second Dutch pedigree with the same hemophilic phenotype. The results indicate that the two Dutch kindreds are related and point to the functional significance of the −20 position for the expression of the human factor IX gene.

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MATERIALS AND METHODS

The patient chosen to test the hypothesis was subject IX-27 from pedigree A. The same person is numbered VI-20 in the updated pedigree by Briët et al. His hemophilia B status is evidenced by the fact that his factor IX activity level was 2.6% at age 1 year, 1% at age 7 years, 1.4% at age 12 years, 7% at age 16 years, 25% at age 18 years, and 37% at age 23 years.

For DNA analysis citrated blood was collected when the patient was 23 years of age, and DNA was isolated according to established procedures from the white cell fraction. For Southern blotting experiments the genomic DNA was digested to completion with restriction enzymes, separated according to size by electrophoresis on a 0.7% agarose gel, and transferred to nylon filters (Gene Screen Plus, New England Nuclear, Boston) according to the instructions of the manufacturer. Restriction fragments specific for the factor IX gene were visualized by hybridization with a factor IX cDNA probe (kindly provided by D. Stafford, University of North Carolina, Chapel Hill) that was radiolabeled with [3H]CTP using a random labeling kit (Boehringer Mannheim, Mannheim, West Germany). Since this cDNA probe contains only 20 basepairs of exon a, a second probe containing the TaqI fragment between basepairs −8 and +312 (numbering by Yoshitake et al.) was used to visualize the restriction fragments that contain the promoter region.

For the cloning of the promoter region, genomic DNA was digested to completion with the restriction enzyme EcoRI. This produces a 12.5 kilobase (kb) fragment of the factor IX gene that contains exon a and includes ~7 kb of 5' flanking sequence, as well as ~5-kb sequence of the first intron flanking exon a. The size of this EcoRI fragment allows direct cloning of the complete digest in the bacteriophage λEMBL3. About 100,000 recombinants were obtained and screened with the factor IX cDNA. Two independent clones that contain the desired promoter fragment were isolated.

No attempt was made to fully sequence the 12.5-kb EcoRI fragment. The analysis has been focused on two adjacent TaqI fragments that contain ~700 bp upstream of the initiation codon and extend ~300 bp downstream. The two fragments were subcloned in phase M13 and sequenced in both directions with a dideoxy sequencing kit (Amersham International, Amersham, UK) using both universal primers and primers specifically designed for the factor IX gene.

While this work was in progress, a new technique became available to amplify specific genomic sequences in amounts sufficient for a direct sequence analysis. This allowed the authors to compare the sequence results obtained from the selected genomic DNA from those from a second patient of the same pedigree, a patient from the other known Dutch family with hemophilia B Leyden (pedigree II'), and from two controls.
PROMOTER MUTATION IN HEMOPHILIA B LEYDEN

Fig 1. Nucleotide sequence of the promoter region of the factor IX gene. The sequence between nucleotide -792 and +312 (numbering as in Yoshitake et al19) has been established in the hemophilia B Leyden patient. Arrows indicate putative transcription start sites, and the T → A mutation at position -20 is indicated by an asterisk. A potential inverted "CCAAT" box is underlined.

For DNA amplification, two oligonucleotides, '5' CATTGAGG-GAGATGGACATT5' and '5'-GGCAAGCATACTCAATG-TAT'-3' were synthesized, and total genomic DNA was amplified as described by Kogan et al. This resulted in 500 to 1,000 ng of the target sequence that was subsequently purified by electrophoresis on a 1% ultralow melting agarose gel (Sigma, St Louis). After melting of the excised fragment, 1 µL was used in a standard dideoxy sequence reaction using either of the two oligonucleotides as a primer. This resulted in a readable sequence from nucleotide -50 to +119.

RESULTS

Our first approach to the analysis of the factor IX gene in hemophilia B Leyden was to check for gross alterations using Southern blotting of total genomic DNA. When using a virtually full-length cDNA probe or a 300-bp genomic probe on HindIII-, EcoRI-, and XbaI-digested DNA, no deviations from the pattern that is obtained with control DNA were detected. This indicates that factor IX coding and intronic sequences are present without large deletions or insertions (data not shown).

Next the authors cloned in λEMBL3 a 12.5-kb EcoRI fragment that contains the putative factor IX promoter. Two independent lambda clones were isolated, and the DNA was digested with various restriction enzymes. The size of the resulting fragments was compared against the published restriction maps8,9 and against a similarly treated clone that was isolated from an unrelated hemophilia B patient carrying a point mutation in his coding sequence (factor IX Hoogeveen, Reitsma et al, unpublished results). This comparison confirmed that no gross DNA alterations have occurred in the 5' part of the factor IX gene.

A computer search with the promoter region of the factor IX gene upstream of the initiation codon shows that this stretch contains only ~300 bp of unique DNA. Further upstream, a 70-bp stretch of DNA is encountered that is similar to sequences flanking globin and IgG genes, which is followed by several kb of DNA homologous with Kpn-like repeats that are ubiquitously present in the human genome. For DNA sequence analysis the authors focused on two adjacent TagI restriction fragments extending from nucleotide -792 to nucleotide +312 (numbering as in Yoshitake et al19) in which the unique portion of the putative promoter region and the first factor IX exon are present (Fig 1).

When the authors compared the nucleotide sequence of the two TagI fragments obtained from the hemophilia B Leyden clones with published sequence data,10 three changes were noted. Two changes in the Kpn repeat region, at positions -598, -597 TC instead of CT and at position -423 a C instead of a T, occur with respect to the Yoshitake sequence.10 To assess the significance of these two changes...
the authors sequenced the promoter region from the factor IX Hoogeveen patient and found the same deviations. This leads the authors to conclude that these two alterations either represent errors in the Yoshitake sequence or neutral polymorphisms.

A final nucleotide change, T → A, was noted at position −20 (Figs 1, 2). Neither of the published sequences nor the factor IX Hoogeveen sequence (Reitsma et al, unpublished) has an adenosine at this position, which indicates that the T → A change is unique for hemophilia B Leyden. Further evidence for the significance of the mutation was obtained by direct sequencing of amplified human genomic DNA. Using this technique, the authors could confirm the mutation in a patient from the same family (patient V-4 in pedigree I) and in a patient from another Dutch pedigree with hemophilia B Leyden (patient X-19 in pedigree II), whereas two additional controls were negative for the change (data not shown).

DISCUSSION

The promoter region of the factor IX gene has been analyzed by Anson et al.9 Using primer extension and S1 nuclease mapping, three start sites of mRNA synthesis were identified, and one of these (position +1) was pinpointed as the major start site (Fig 1). Inspection of the sequence upstream of the start site does not indicate the presence of known signal sequences with the possible exception of an inverted “CCAAT” box at position −92 (indicated in Fig 1). Particularly striking is the absence of a canonical “TATA” box sequence ~30 bp upstream of the putative start site, an observation that has also been made in other liver-derived coagulation proteins.10

Given this incomplete understanding of which sequences signal accurate and efficient initiation of transcription in the human factor IX gene, it is difficult to assess by analogy the significance of the nucleotide change at position −20. One report that deals with the effect of promoter mutations on transcription efficiency of the fibrinogen gene points to the importance of position −20.11 In contrast, a large survey of promoter mutations in the β-globin gene suggests that the −20 position is of little importance for efficient transcription of this gene.12

If the authors assume that the nucleotide at position −20 is indeed crucial for efficient factor IX transcription and explains the absence of normal factor IX gene expression, the causes of the direct or indirect effect of testosterone remain enigmatic. It may be that a second change has occurred that has escaped the analysis or that the factor IX promoter has the intrinsic potential of being regulated by testosterone, for instance through the use of an alternative promoter, and that this route is activated when a promoter mutation occurs.

The two Dutch hemophilia B Leyden families originate from the same rural area in the eastern part of Holland, but even after an extensive genealogic study the two families could not be linked.22 The results reported here add further evidence to the notion that the two families are indeed related.

In conclusion, the authors’ current hypothesis is that a mutation at position −20 of the factor IX gene is responsible for hemophilia B Leyden. Other changes cannot be excluded, and further studies that assess functional properties of the factor IX gene promoter in expression systems may corroborate the significance of the findings.

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REFERENCES

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