Two Novel Point Mutations Correlate With an Altered Developmental Expression of Blood Coagulation Factor IX (Hemophilia B Leyden Phenotype)

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Hemophilia B Leyden is characterized by low levels of factor IX antigen and activity before the age of 15 years, whereas after puberty factor IX levels rise at a rate of about 5% per year. Two distinct point mutations (deletion of A, A → G) were identified at position +13 of the factor IX gene of a Greek and an American patient with hemophilia B Leyden. The nucleotide changes have occurred 32 basepairs downstream of a previously reported point mutation in a Dutch kindred with the same hemophilic phenotype. The results point to the importance of sequences surrounding the putative start site for the constitutive expression of the factor IX gene and to the possible significance of an imperfect direct repeat of DNA.

A SUBGROUP of unrelated hemophilia B patients is characterized by a similarly altered developmental expression of blood coagulation factor IX (so-called hemophilia B Leyden). In these patients plasma factor IX levels are ≤1% of normal before puberty, whereas thereafter factor IX activity and antigen levels steadily rise in a 1:1 ratio to a maximum of 50% to 60%.

Recently the nucleotide sequence of an ~700-bp long promoter fragment was determined in patients from two, most likely related, Dutch pedigrees with hemophilia B Leyden. The analysis revealed in patients from both pedigrees the same single-point mutation (T → A) at position −20 of the factor IX gene. This led to the hypothesis that hemophilia B Leyden results from a promoter mutation and that sequence elements very close to the putative start site of transcription are crucial for constitutive factor IX transcription. Moreover, the results suggested that single-point mutations may suffice to switch from constitutive to steroid hormone-dependent gene expression.

To obtain further, independent support for this hypothesis, the factor IX gene promoter has been analyzed in two additional, unrelated families with a hemophilia B Leyden phenotype. This analysis reveals two (distinct) single-point mutations at position +13 of the factor IX gene (ie, just within the putative transcribed region). The results also indicate that an imperfect “direct repeat” of DNA around the start site(s) for transcription may be important for normal factor IX gene expression.

MATERIALS AND METHODS

The first family studied is of Greek origin, and the hemophilia B Leyden status in affected individuals has been described in detail. DNA was obtained from the white cells of a patient (subject V-3′), and the factor IX gene promoter was cloned and sequenced according to the strategy outlined for the Dutch families. In addition, the findings were confirmed in the hemophilia patient and in his mother (who is an obligate carrier of the defective allele) using direct sequencing of amplified genomic DNA.

The second family that was investigated is of Armenian descent and lives in the United States. The upper panel of Fig 1 shows the pedigree of this family, which included only one patient with a well-documented Leyden type of hemophilia B (subject III-1, lower

Fig 1. Upper panel: Pedigree of the American family with hemophilia B Leyden. The proband is individual III-1. As a young boy he had fairly frequent hemorrhages, but since puberty he seldom bleeds. Subject II-3 (hatched) was said to have been a bleeder as a child but was only tested at the age of 39 years when his factor IX was 42%. II-1 (half hatched) is the only carrier in this limited kindred whose factor IX level was 79%. Lower panel: Factor IX activity level in patient III-1 as a function of age. Before the age of 14 years no factor IX was measurable, whereas at the age of 18 years it had risen to a level of 24% and has remained at that level thereafter.

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panel of Fig 1). For the analysis of this family, DNA from subject III-1 was available. No attempt was made to determine the nucleotide sequence of the factor IX promoter region on cloned DNA of the patient. Instead, the analysis was limited to determining the nucleotide sequence of the factor IX gene between bases −50 and +119 (numbering as in Yoshitake et al) using the direct sequencing strategy mentioned above.

RESULTS

The analysis of the Greek patient focused on two adjacent TaqI fragments of the factor IX gene that contain ~700 bp upstream of the putative initiation codon and extend ~300 bp downstream. Analysis of the nucleotide sequence revealed a single-point mutation at position +13, where one adenosine was deleted in the sequence . . . CAAT . . . (data not shown). The single base deletion could be confirmed in DNA from the patient and from the mother of the patient by direct sequencing of amplified genomic DNA, as is shown for the opposite strand in Fig 2.

Given the result with the Greek patient and the previous experience with the Dutch family, the analysis of the American patient was limited to the nucleotide sequence between positions −50 and +119. This analysis again revealed a single-point mutation (A → G) at position +13, as is shown for the opposite strand in Fig 3.

Further support for the significance of the findings was obtained when six normal alleles were analyzed between bases −50 and +119. This analysis did not reveal a deviation from the reported sequences, indicating that sequence anomalies are not common in the factor IX gene promoter.

DISCUSSION

The findings in the Greek and American patients with hemophilia B Leyden confirm the hypothesis that this type of hemophilia B may result from single-point mutations in the promoter region of the factor IX gene. Surprisingly, however, the two distinct mutations have occurred at a position 32 basepairs downstream of the mutation that was identified in the Dutch families (Fig 4, upper panel). Moreover, the mutations are not in the promoter per se but in the putative, transcribed, untranslated portion of the gene.
sequences surrounding the transcription start site(s) can be interpreted as an imperfect, direct repeat. The three point mutations occur in the interrupted "consensus" ACTTT(N)(N)NACAA.

At the moment the functional significance of the "consensus" sequence is not clear. With exception of the TATA box, sequences surrounding the start site of transcription are considered to be of minor importance for appropriate transcription.9 These findings can be exemplified by the β-globin gene where point mutations outside of the TATA- or CCAAT-box do not significantly influence the rate of transcription in vitro.10

Notable exceptions to the finding that there is no function for sequences surrounding the start site of messenger RNA (mRNA) synthesis are given by the chicken conalbumin and ovalbumin genes. Dierich et al11 showed for these two genes that promoter sequences close to the TATA-boxes (between nucleotide −44 to +62 for conalbumin, and nucleotide −56 to −1 for ovalbumin) are important for tissue-specific initiation of transcription, and these authors postulated the existence of cell-specific factors that interact with these sequence elements. Maybe the factor IX gene is also one of a subset of genes for which tissue specificity resides in sequences near the transcription start site that even may extend into the transcribed portion of the gene.

If the point mutations are interpreted as interfering with constitutive factor IX gene expression, which is most evident from the severe bleeding tendency before puberty in affected males, it still remains unexplained what causes the (testosterone-dependent) factor IX increase after puberty. It seems unlikely that a second mutation conferring testosterone dependency to the factor IX gene has arisen independently in the three families. More likely the testosterone effect may be intrinsic to the factor IX gene but may only become manifest, for instance through the use of an alternative promoter

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**Fig 3.** Autoradiogram illustrating the nucleotide change in the American patient with hemophilia B Leyden. As in the left panel, the sequence of the opposite strand of the factor IX gene promoter was obtained after direct sequencing of amplified genomic DNA. The asterisk marks the mutation (T → C in the opposite strand) in the hemophilia patient.

Since the point mutations in the Greek and American families occurred downstream of the mutation in the Dutch family, a search was made for sequence similarities in the two regions where the mutations have occurred. The sequence alignment shown in Fig 4 (lower panel) gives the maximal identity between the two regions. It shows that the

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**Fig 4.** Upper part: Nucleotide sequence of the promoter region of the human factor IX gene. The nucleotide changes observed in the Greek, American, and Dutch patients are boxed. The putative major start site of transcription is indicated by a closed circle, two putative minor start sites by an open circle.13 A potential inverted CCAAT box is underlined. The amino acids of the first exon of the factor IX gene are given in single letter code. Nucleotide numbering is as in ref 8. Lower part: Alignment of the regions in which the point mutation in hemophilia B Leyden patients were found. To maximize the similarity, two gaps were allowed in each of the sequence elements. The positions of the point mutations are indicated by an asterisk.
site, after the normal promoter is down mutated. At the moment nothing is known about the sequence requirements for testosterone dependency of gene expression so that candidate control elements and/or alternative promoter sites cannot be proposed on the basis of sequence comparisons alone but could follow from future experimentation.

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