Hemophilia B Leyden: Substitution of Thymine for Guanine at Position −21 Results in a Disruption of a Hepatocyte Nuclear Factor 4 Binding Site in the Factor IX Promoter

By Marlene J. Reijnen, Kathelijne Peerlinck, Dieda Maasdam, Rogier M. Bertina, and Pieter H. Reitsma

Hemophilia B Leyden is an X chromosome–linked bleeding disorder characterized by an altered developmental expression of blood coagulation factor IX. This form of hemophilia B has been found to be associated with a variety of single point mutations in the factor IX promoter region. We now describe a novel point mutation, T→G at position −21, in two related patients with the hemophilia B Leyden phenotype. This mutation lies within the factor IX promoter region (−40 to −9) that contains overlapping binding sites for hepatocyte nuclear factor 4 (HNF-4) and androgen receptor. Transient transfection assays in HepG2 cells show that the −21 mutation causes a significant reduction in factor IX promoter activity. Gel mobility shift assays and transient cotransfection experiments revealed that the HNF-4–binding site but not the androgen-responsive element is disrupted by the −21 mutation. A comparison of the −21 mutation with the previously described −20 T→A mutation (associated with the hemophilia B Leyden phenotype) and −28 G→C mutation (associated with severe hemophilia B throughout life) was made. It shows that the −21 mutation reduced HNF-4 binding and transactivation to a similar level as the −20 mutation, whereas the −26 mutation completely abolished HNF-4 binding and transactivation. Mobility shift experiments indicate that there was no significant difference in binding affinity of recombinant androgen receptor protein for oligonucleotides containing wild-type and −21 or −20 mutated DNA. The binding affinity for the oligonucleotide containing the −26 mutation was twofold lower. The results indicate that the disruption of the HNF-4–binding site by the −21 T→G mutation is the cause of the bleeding disorder in these two patients. This study adds further support for the notion that the recovery from hemophilia at puberty may not only be related to an intact androgen-responsive element but also to the degree of disruption of the HNF-4–binding site.

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trifugation and tested for fIX clotting activities and antigen levels. fIX clotting activities were measured by one-stage assay and adapted on an automate in coagulation testing (ACL-810, IL, Milan, Italy). fIX antigen levels were assayed by the ELISA method and measured automatically on a densitometric analysis microwell system (Organon Teknika, Boxtel, The Netherlands). DNA was prepared from the white cells. A DNA fragment (~230 bp) of the putative fIX promoter region spanning nucleotides -193 to +40 was amplified by the polymerase chain reaction (PCR).

**Fig 1.** (A) Pedigree of the Belgian family with hemophilia B Leyden. Four generations of the family are shown. The two patients affected with hemophilia B Leyden are III-5 and III-8 (solid squares). Half-solid circles denote carriers. (B) The fIX activity levels in each of the patients (III-5 and III-8) in relation to age. Before puberty, no fIX was measurable. (C) Sanger dideoxy sequence analysis of the putative factor IX (fIX) promoter region of the two patients, III-5 (track 2) and III-8 (track 3), and of one normal male (track 1). The arrows mark the mutation T→G at -21 in the patients.

**Transient transfection.** The wild-type, the -20 T→A, and the -26 G→C mutated fIX promoter-CAT constructs have been described previously. The -21 mutated fIX promoter-CAT plasmid, designated -192(-21T→G)CAT00.1, was similarly constructed. It contains the -21 T→G mutated human fIX promoter region, spanning nucleotides -192 to +38, cloned immediately upstream of the CAT gene. Relevant portions of the resulting reporter construct were verified by DNA sequencing. Transient transfection assays were performed in HepG2 and HeLa cells seeded into 94-mm diameter Petri dishes. DNA (20 μg fIX-CAT, 2 μg of HNF-4 expression vector [pLEN4St1] and 2 μg of β-galactosidase expression vector [pCH I 10]) were transfected into the cells as described earlier.

**Gel mobility shift assay.** The analysis of protein-DNA complexes by mobility shift assays and the preparation of crude nuclear extracts from rat livers and HepG2 cells were as described previously. Labeled oligonucleotides containing the wild-type or -21 mutated fIX promoter region (-40 to -9: Table I) were incubated with nuclear extracts from rat liver, HepG2 cells, or in vitro translated HNF-4 either in the absence or presence of competitor DNA. Bacterially expressed androgen receptor-protein A fusion protein (a gift from Dr P. De Vos) was used in mobility shift assays of androgen-responsive element containing oligonucleotides (oligo “wt”, m-21, m-20, m-26 and ARE; Table 1). These experiments were performed exactly as described by De Vos et al. For competition experiments, increasing amounts of double-stranded oligonucleotide competitors (Table 1) were added to a constant amount of nuclear extract and 32P-labeled probe DNA. Relative amounts of protein-DNA complexes were determined either by densitometric analysis (IKB Ultrascan XL densitometer; Pharmacia, Uppsala, Sweden) of autoradiographs or by counting...
the amount of radioactivity (protein bound and free probe) from the dried gels on the Phosphor Imager (Molecular Dynamics, Sunnyvale, CA) using the Image Quant analysis system. Shown is the average of three experiments.

RESULTS

The factor IX coagulant activity levels (fIX:C) in the two patients with hemophilia B Leyden (III-5 and III-8) were measured up to the age of 19 and 22 years, respectively (Fig 1B). The patients had a clinical history compatible with severe hemophilia B in the prepuberty years (fIX:C levels below 1% of normal). One of the patients needed prophylactic treatment for frequent hemorrhages. After onset of puberty, the fIX:C levels gradually rose to 12% to 14% and are still rising. With the rise of fIX:C levels, the spontaneous bleeding tendency disappeared. In a recent blood sample of these two hemophiliacs, factor IX antigen was found to be ~10%, with factor IX antigen/activity ratios of 0.96 and 0.71, respectively (indicating the synthesis of fully active factor IX molecules).

A variety of single point mutations in the putative fIX promoter region has been associated with hemophilia B Leyden. Using the strategy of PCR amplification and the Sanger direct sequencing analysis, we determined the sequence of the fIX promoter region of these two patients with hemophilia B Leyden (Fig 1C, III-5 and III-8). A novel point mutation, T→G at position -21, of the fIX gene was detected. This mutation, in addition to a normal allele, was also present in maternal DNA (II-3 and II-4) and DNA prepared from the sister (III-9) of patient III-8, thus confirming their carrier status.

To determine whether the -21 T→G mutation impairs transcription from the fIX promoter, we analyzed the transcriptional activity of the wild-type and -21 mutated promoters. The fIX promoter–CAT constructs containing wild-type and -21 T→G mutated fragments (extending from -192 to +38) were tested for expression in transiently transfected HepG2 and HeLa cells. In agreement with previous observations, the wild-type promoter of the human f IX gene was capable of directing CAT transcription in HepG2 cells but not in HeLa cells (Fig 2). When the transfactions in HepG2 were carried out with the -21 T→G mutated construct (-192(-21T→G)CATO.O.I), only a background level of CAT activity was found. Similar experiments as described for the -21 mutation were previously done for the -20 T→A and -26 G→C mutated fIX promoters. Like the -21 mutation, they also produced only background levels of CAT activity in HepG2 cells (Fig 2A).

Because the T→G mutation at -21 lies within a region that contains a HNF-4-binding site and an overlapping androgen-responsive element, we tested whether this mutation disrupts either of them. Oligonucleotides containing the -40 to -9 region of the fIX promoter (wild type: oligo “wt” and -21 T→G mutated: oligo m-21) were used in gel mobility shift assays (Table 1). HNF-4 protein, either from HepG2 cells (Fig 3A, lanes 1 and 3) or translated in vitro from HNF-4 cDNA (lane 8), bound the labeled oligo “wt” very well. When the experiments were carried out with labeled oligo m-21, reduced binding of HNF-4 to -21 mutated site was found (lane 2). The labeled HNF-4-DNA complex was competed effectively by 40-fold molar excess of unlabeled oligo “wt” (lanes 4 and 9) and to a lesser extent by oligo m-21 (lanes 5 and 10). To confirm that indeed HNF-4 binding is involved, we incubated the mobility shift reaction mixtures with specific antibodies to HNF-4.11 This resulted in a “supershift” of the complex (lane 6). The addition of anti–NF-1 antibody (lane 7, negative control) had no effect. Additional competition experiments were performed with increasing amounts of unlabeled competitor DNA (oligo “wt”, m-21, m-20 or m-26; Table 1) added to a constant amount of rat liver nuclear extract and labeled oligo “wt” (Fig 3B). The experiments were repeated three times. Both the -21 mutation and the -20 mutation disrupt the HNF-4-binding site to a similar level (~16 times more mutant DNA than wild-type DNA was needed to reduce the formation of labeled protein-DNA complex by 50%). The -26 mutated oligonucleotide (m-26) competed only marginally for HNF-4 binding. Similar observations were made with HepG2 nuclear extracts (data not shown). These results indicate that the mutation at -21 interferes with HNF-4 binding to the fIX promoter. Moreover, although the -21 mutation and the -20 mutation partially reduce HNF-4 binding, the -26 mutation seems to completely disrupt the HNF-4-binding site.

To determine whether the androgen-responsive element was also disrupted by the -21 mutation, we performed gel mobility shift assays with a recombinant androgen receptor–protein A fusion protein.12 When the experiments were carried out with labeled oligo “wt” as probe, only a small fraction of the probe was retarded (Fig 4A, lanes 1 and 2). In contrast, the fusion protein bound very well to a probe containing the well-characterized androgen-responsive element from the rat prostatic binding protein gene (oligo ARE, lanes 3 and 4). Therefore, in an attempt to establish a difference in binding of the fusion protein to the wild-type and -21 mutated fIX promoter region, we performed competition experiments with oligo ARE as probe and increasing concentrations of unlabeled oligo “wt” and oligo m-21 as competitors (Fig 4B). Compared with the competition with oligo ARE, large amounts (300- to 400-fold mol/L excess) of unlabeled oligo “wt” and oligo m-21 were required to reduce the formation of labeled protein DNA complex by 50% (Fig 4B,C). There was no significant difference in competition for fusion protein binding between oligo “wt” and oligo m-21. When the experiments were carried out with

<table>
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<th>Table 1. Oligonucleotides Used in Gel Mobility Shift Assays</th>
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<tr>
<td><strong>Sequence</strong></td>
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<tr>
<td>Human factor IX gene</td>
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<tr>
<td>“wt” ATACAGCTACGTGCTGTTCTTGTGTTCTACAACGTA</td>
</tr>
<tr>
<td>m-21 ATACAGCTACGTGCTGTTCTTGTGTTCTACAACGTA</td>
</tr>
<tr>
<td>m-20 ATACAGCTACGTGCTGTTCTTGTGTTCTACAACGTA</td>
</tr>
<tr>
<td>m-26 ATACAGCTACGTGCTGTTCTTGTGTTCTACAACGTA</td>
</tr>
<tr>
<td>Rat prostatic binding protein gene AREGATCATGACGTGGTCTCTCAAGTC</td>
</tr>
<tr>
<td>Chicken ovalbumin gene COUPCTCATGGCTGCGAAGTGC AACTCT</td>
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The sequence and location of the top strand of the oligonucleotides used in competition binding assays are given. Mutated nucleotides are indicated by lower case letters.
Fig 2. Effects of the −21, −26, and −20 mutations on fX promoter activity in HepG2 cells (A) and HeLa cells (B). Plasmid constructs with the CAT reporter gene under control of wild-type (−192CATOO.1), −21 T→G mutated, −26 G→C mutated, and −20 T→A mutated fX promoters were tested for CAT activity in the absence (■) or presence (□) of the HNF-4 expression vector pLEN4S.11 CATOO.1 was the promoterless control plasmid. CAT activity was normalized to the activity of a β-galactosidase internal standard. Shown is the average normalized CAT activity of three experiments.
unlabeled oligo m−20 as competitor, similar results were obtained (Fig 4C). The affinity of the fusion protein for oligo m−26 was lower since a twofold higher concentration of DNA was needed to reduce the formation of labeled protein DNA complex by 50%. No competition was observed with excess unlabeled oligonucleotide containing the COUP-TF-binding sequence (COUP). These results indicate that the HNF-4-binding site but not the androgen-responsive element is disrupted by the −21 T→G mutation.

The functional consequence of the reduction in HNF-4 binding to the −21 mutated FIX region was analyzed by transient cotransfection experiments in HepG2 and HeLa
Fig 4. Analysis of androgen receptor binding to wildtype and mutated factor IX promoter regions. (A) Mobility shift assay with androgen receptor–protein A fusion protein and labeled oligo “wt” (lanes 1 and 2) or labeled oligo ARE (lanes 3 and 4) as probes. (B) Competition mobility shift assay done with labeled oligo ARE as probe and fusion protein (150 fmol of labeled DNA and 0.4 μg of androgen receptor–protein A fusion protein) using oligo “wt” and oligo m – 21 as competitors. Binding was inhibited with an increasing mol/L excess of unlabeled oligo “wt” and oligo m – 21 (40-, 100-, 200-, 400-, and 800-fold mol/L excess). (C) Graph of the competition analysis done with androgen receptor–protein A fusion protein using wild-type (oligo “wt”) and mutated (oligos m – 21, m – 20, and m – 26) factor IX promoter sequences, the COUP-TF–binding site of the chicken ovalbumin promoter (COUP) and the androgen-responsive element of the rat prostatic binding protein (oligo ARE) as competitors (Table 1). Increasing amounts of unlabeled oligonucleotides “wt”, m – 21, m – 26, m – 20, and ARE were added to a mixture of a fixed amount of end-labeled oligonucleotide ARE as probe and androgen receptor–protein A fusion protein. The amounts of radioactivity (protein bound and free probe) were measured on a Phosphor Imager using the Image Quant analysis system. The values of the formation of labeled androgen receptor–DNA complex throughout is shown relative to the value of labeled androgen receptor–DNA complex in the absence of competitor, which is arbitrarily set at 100. Shown is the average of three experiments.
cells. In these experiments, an expression vector for HNF-4 (pLEN4S\textsuperscript{11}) was cotransfected with the reporter constructs described above. The results presented in Fig 2A,B show that HNF-4 transactivates the wild-type reporter construct (−192CATOO.1) in both cell types quite well. Compared with the wild-type construct, the −21 mutated plasmid (−192−21 T→GJCATOO.1) was expressed at a significant lower level (18% in HepG2 cells and 15% in HeLa cells) in response to HNF-4 transactivation. Similar observations were made for the previously reported −20 T→A mutated promoter (17% in HepG2 cells and 20% in HeLa cells), whereas no transactivation was observed of the −26 G→C mutated promoter.

**DISCUSSION**

A T→G base change at position −21 was detected in the fIX promoter region of two related patients with the hemophilia B Leyden phenotype. The mothers of the patients also carried the defective allele suggesting that the original mutation has occurred in one of their parents or other forebears.

The T→G mutation at −21 in both patients is associated with severe bleeding symptoms and plasma fIX levels below 1% of normal during childhood. The severity of the fIX deficiency in these two patients is similar to those observed in the patients with mutations at position −20. Interestingly, the residues at −22, −21, and −20 (all thymine residues) are conserved in various mammalian species (ie, human, macaque, dog, rat, and mouse\textsuperscript{17}). It seems that the residues at −21 and −20 are critical for transcription initiation.

The causal relationship between the −21 T→G base change and the hemophilia B Leyden phenotype was demonstrated by transient transfection analysis and in vitro binding studies. The mutation, which lies within overlapping binding sites for androgen receptor and HNF-4 in the fIX promoter, causes a significant reduction in promoter activity. We showed that the T→G mutation at −21 interferes with the binding of HNF-4 to its recognition site (between −34 and −10). Moreover, we show that although HNF-4 transactivates the wild-type fIX promoter quite well, it could activate the −21 T→G mutated promoter only to a limited extent. The data presented here indicate that the disruption of the HNF-4–binding site by the −21 T→G mutation is the cause of the severely impaired (hepatic) fIX gene expression in these patients during childhood.

These results add further support for the notion that HNF-4 plays a dominant role in controlling fIX gene expression in normal individuals. In fact, the observation that fIX promoter constructs are very efficiently activated by HNF-4 in the nonhepatic HeLa cells suggest that HNF-4 is the major determinant of the liver-specific production of fIX.

It was recently reported that the fIX promoter contains a functional androgen-responsive element between −40 and −9.\textsuperscript{7} These studies demonstrated that four copies of the wild-type fIX promoter region (−42 to −17) confer androgen responsiveness to a herpes simplex thymidine kinase (tk) promoter in a heterologous system. When the −26 G→C mutation was present no transactivation was observed, whereas the −20 T→A mutation did not affect transactivation in HeLa cells. When we tested this candidate androgen-responsive element in gel mobility shift assays, the binding affinity of the androgen receptor (ie, recombinant androgen receptor–protein A fusion protein) for this site was rather low when compared with the androgen-responsive element from the rat prostatic binding protein gene. Furthermore, transient cotransfection experiments with several different androgen receptor coding plasmids in HepG2 cells showed that this potential androgen responsive element in the fIX promoter responded only poorly to the activation by androgens.\textsuperscript{18} Although such transactivation experiments may not reflect the in vivo situation, this marginal response to androgens is not comparable with the significant increase in fIX expression in patients with hemophilia B Leyden after puberty.

Assuming that the putative fIX androgen-responsive element is functional in the fIX promoter, it is interesting to note that the −26 mutation causes only a minor difference in binding affinity of the androgen receptor–protein A fusion protein. The fact that this mutation not only disrupts the androgen-responsive element but also completely blocks HNF-4 binding suggests a synergistic interaction between androgen receptor and HNF-4. The observation that neither of the hemophilia B Leyden–like mutations at −21 and at −20 completely eliminates HNF-4 binding and transactivation suggests that remnant HNF-4 binding is required for the androgen-mediated response after puberty. Both androgen receptor and HNF-4 are members of the steroid hormone receptor superfamily, a group of ligand-dependent transcription factors that possess a high degree of similarity in their DNA-binding domains. It is therefore possible that HNF-4 forms heterodimers with the androgen receptor, as has been seen for the thyroid hormone receptors and the retinoic acid receptors.\textsuperscript{19,20}

**ACKNOWLEDGMENT**

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Hemophilia B Leyden: substitution of thymine for guanine at position -21 results in a disruption of a hepatocyte nuclear factor 4 binding site in the factor IX promoter

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