Hemophilia B Leyden: Substitution of Thymine for Guanine at Position $-21$ in the Factor IX Promoter

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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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PATIENTS, MATERIALS, AND METHODS

Patients. The family studied is of Belgian origin (Fig 1A). Patient III-5 had regular spontaneous hemarthroses at least once weekly, usually in his ankles and right elbow and occasionally in his knees till the age of 15 years. From that age onward, spontaneous hemarthroses no longer occurred, and the patient only needed transfusions after accidents or prophylactically before minor surgery. Patient III-8 had very frequent, recurrent spontaneous hemarthroses as a child, especially in the right elbow. At least weekly transfusions were needed till the age of 14. Thereafter, the frequency of spontaneous hemarthroses diminished, and at the age of 18, the spontaneous bleeding tendency disappeared.

Blood samples, collected in 0.1 volume of 0.1 mol/L sodium citrate, were obtained from the patients (III-5 and III-8) and the family members (II-3, II-4, and III-9). Plasma was obtained by cen-
trifugation and tested for FIX clotting activities and antigen levels. FIX clotting activities were measured by one-stage assay and adapted on an automated 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). The sequence and location of the oligonucleotide primers used in the PCR amplification are 5' TGTACTTTGGTACAACTA 3' and 5'ACGCGCTGATACCTTGCGTGC 3'. Amplified DNA obtained from two separate amplification reactions was purified by agarose gel electrophoresis and sequenced using the Sanger direct sequencing strategy. Sequencedata were obtained by sequence analysis of both the sense and antisense strands of DNA. The analysis was limited to determining the nucleotide sequence of the FIX promoter region between nucleotide -192 and +40.

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)CAT001, 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 [pCH110] were transfected into the cells as described earlier. The CAT activity of each construct, which was normalized for β-galactosidase activity, was determined in three independent transfection experiments. Shown is the average normalized CAT activity of three experiments.

**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 I). These experiments were performed exactly as described by De Vos et al.

For competition experiments, increasing amounts of double-stranded oligonucleotide competitors (Table I) were added to a constant amount of nuclear extract and labeled probe DNA. Relative amounts of protein-DNA complexes were determined either by densitometric analysis (LKB 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, Sun-
yvale, 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 se-
vere hemophilia B in the prepuberty years (fIX:C levels be-
low 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 bleed-
ing 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, re-
spectively (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 se-
quence 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 pre-
pared 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 tran-
scriptional activity of the wild-type and −21 mutated pro-
moters. 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 transfect-
ated HepG2 and HeLa cells. In agreement with previous
observations, the wild-type promoter of the human
fIX gene was capable of directing CAT transcription in
HepG2 cells but not in HeLa cells (Fig 2). When the trans-
fections in HepG2 were carried out with the −21 T→G
mutated construct (−192(−21T→G)CATOO.I), only a
background level of CAT activity was found. Similar experi-
ments as described for the −21 mutation were previously
done for the −20 T→A and −26 G→C mutated fIX pro-
moters. 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 an-
drogen-responsive element, we tested whether this muta-
tion 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 la-
beled oligo m−21, reduced binding of HNF-4 to −21 mu-
tated site was found (lane 2). The labeled HNF-4-DNA
complex was competed effectively by 40-fold molar excess
of unlabelled 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)16
had no effect. Additional competition experiments were
performed with increasing amounts of unlabelled 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) com-
peted only marginally for HNF-4 binding. Similar observa-
tions 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. More-
over, although the −21 mutation and the −20 mutation
partially reduce HNF-4 binding, the −26 mutation seems to
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 recep-
tor–protein A fusion protein.17 When the experiments were
conducted 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 con-
taining 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 differ-
ce in binding of the fusion protein to the wild-type and
−21 mutated fIX promoter region, we performed competi-
tion experiments with oligo ARE as probe and increasing
concentrations of unlabelled 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 unlabelled 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 com-
petition for fusion protein binding between oligo “wt” and
oligo m−21. When the experiments were carried out with

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Table 1. Oligonucleotides Used in Gel Mobility Shift Assays

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human factor IX gene</td>
<td></td>
</tr>
<tr>
<td>&quot;wt&quot; ATACAGCTAGCTGTTGACTTGACAAGTCA</td>
<td>−40 to −9</td>
</tr>
<tr>
<td>m−21 ATACAGCTAGCTGTTGACTTGACAAGTCA</td>
<td>−40 to −9</td>
</tr>
<tr>
<td>m−20 ATACAGCTAGCTGTTGACTTGACAAGTCA</td>
<td>−40 to −9</td>
</tr>
<tr>
<td>m−26 ATACAGCTAGCTGTTGACTTGACAAGTCA</td>
<td>−40 to −9</td>
</tr>
<tr>
<td>Rat prostatic binding protein gene</td>
<td></td>
</tr>
<tr>
<td>ARE GATCATGACGCTGTATCAAGAC</td>
<td>coral II</td>
</tr>
<tr>
<td>Chicken ovalbumin gene</td>
<td></td>
</tr>
<tr>
<td>COUP CTAGCTGCTGAAAGTCCTAAGCCT</td>
<td>−90 to −65</td>
</tr>
</tbody>
</table>

The sequence and location of the top strand of the oligonucleotides used in com-
petition binding assays are given. Mutated nucleotides are indicated by lower case
letters.

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Fig 2. Effects of the −21, −26, and −20 mutations on flX 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 flX promoters were tested for CAT activity in the absence (□) or presence (■) of the HNF-4 expression vector pLEN4S. 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.
Fig 3. Analysis of the effect of the $-21$ T$\rightarrow$G mutation on the binding affinity for hepatocyte nuclear factor 4 (HNF-4) by gel mobility shift assays. (A) Crude nuclear extract from HepG2 cells (lanes 1 through 7) or in vitro translated HNF-4 (lanes 8 through 10) was incubated with 150-fmol labeled oligonucleotide “wt” (lanes 1 and 3 through 10) or oligonucleotide m$-21$ (lane 2) as probes. The assay was done in the absence (−), lanes 1 through 3, 6, 7, and 8) or presence (+) of 40-fold mol/L excess of unlabeled oligonucleotides “wt” (lanes 4 and 9) or m$-21$ (lanes 5 and 10) or in the presence of antiserum (lane 6, anti-HNF-4; lane 7, anti-NF1). (B) Competition mobility shift assays with wild-type and mutated ($-21$, $-20$, and $-26$) factor IX promoter sequences ($-40$ to $-9$) using crude rat liver nuclear extracts. Increasing amounts of unlabeled oligonucleotides “wt”, m$-21$, m$-26$, and m$-20$ were added to a constant amount of end-labeled oligonucleotide “wt” as probe and rat liver nuclear extracts. Binding was quantitated by densitometric scans (LKB Ultroscan XL densitometer) of the autoradiograms. The values of the formation of labeled HNF-4–DNA complex throughout is shown relative to the value of labeled HNF-4–DNA complex in the absence of competitor, which is arbitrarily set at 100. The experiments were carried out three times. The values reported represent averages of the 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$\rightarrow$G mutation.

The functional consequence of the reduction in HNF-4 binding to the $-21$ mutated flX 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\(^{11}\)) 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 \((-192\text{CATOO}0.1\) in both cell types quite well. Compared with the wild-type construct, the \(-21\) mutated plasmid \((-192\text{[21}T\rightarrow G]\text{CATOO}.1\) 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 \(-20 T\rightarrow A\) mutated promoter (17% in HepG2 cells and 20% in HeLa cells), whereas no transactivation was observed of the \(-26 G\rightarrow C\) mutated promoter.

**DISCUSSION**

A T\(\rightarrow\)G base change at position \(-21\) was detected in the flX 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\(\rightarrow\)G mutation at \(-21\) in both patients is associated with severe bleeding symptoms and plasma flX levels below 1% of normal during childhood. The severity of the flX 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\(^{17}\)). It seems that the residues at \(-21\) and \(-20\) are critical for transcription initiation.

The causal relationship between the \(-21\) T\(\rightarrow\)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 flX promoter, causes a significant reduction in promoter activity. We showed that the T\(\rightarrow\)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 flX promoter quite well, it could activate the \(-21\) T\(\rightarrow\)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\(\rightarrow\)G mutation is the cause of the severely impaired (hepatic) flX gene expression in these patients during childhood.

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

It was recently reported that the flX promoter contains a functional androgen-responsive element between \(-40\) and \(-9.7\). These studies demonstrated that four copies of the wild-type flX promoter region (\(-42\) to \(-17\)) confer androgen responsiveness to a herpes simplex thymidine kinase (tk) promoter in a heterologous system. When the \(-26\) G\(\rightarrow\)C mutation was present no transactivation was observed, whereas the \(-20 T\rightarrow A\) 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 flX promoter responded only poorly to the activation by androgens.\(^{18}\) Although such transactivation experiments may not reflect the in vivo situation, this marginal response to androgens is not comparable with the significant increase in flX expression in patients with hemophilia B Leyden after puberty.

Assuming that the putative flX androgen-responsive element is functional in the flX 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 \(-20\) 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.\(^{19,20}\)

**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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