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Twenty-Four Novel Hemophilia B Mutations Revealed by Rapid Scanning of the Whole Factor IX Gene in a French Population Sample

Abstract

Full scanning of the factor IX gene by means of denaturing gradient gel electrophoresis enabled us to determine the molecular defects in 48 out of 49 hemophiliacs and to evaluate the spectrum of factor IX mutations in the French population. Our results further document the high molecular heterogeneity of the disease and the efficiency of this rapid screening method for disease-causing mutations. This direct approach, which is based on computer-aided analysis of the whole coding, promoter and exon-flanking factor IX gene sequences, proved to be helpful for carrier detection and prenatal diagnosis in most hemophilia B families, including sporadic cases. Moreover, we were able to identify 24 novel molecular defects of various natures in the factor IX gene.

Key Words
Hemophilia B
Factor IX gene
PCR
DGGE

Introduction

Hemophilia B is an X-linked recessive hemorrhagic disorder caused by a deficiency or abnormality of clotting factor IX. Human factor IX is a vitamin-K-dependent multidomain glycoprotein composed of 415 amino acids. It is initially synthesized in the liver as a precursor molecule, some 40 residues longer at its N-terminus than mature factor IX. The gene for factor IX lies on chromosome X at Xq27 and its entire 33-kb sequence is known [1]. It contains 8 exons that encode 6 major domains of the protein. Exon 1 encodes the 28-residue hydrophobic signal peptide, which targets the protein for secretion from the hepatocyte into the blood stream. Exons 2 and 3 encode a propeptide and a Gla domain, the latter containing 12 γ-carboxyglutamyl residues. The two epidermal growth factor (EGF)-like domains (residues 47–84 and 85–127) are encoded by exons 4 and 5 and the activation peptide region is encoded by exon 6. Exon 7 encodes part of the catalytic domain that includes the active-site His-221 residue. The rest of the protease domain is encoded by exon 8.

Detection of restriction site polymorphisms within or closely linked to the factor

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IX gene has given rise to a diagnostic approach based on linkage analysis in hemophilia B families. The feasibility of indirect diagnosis depends on the availability of informative DNA markers and cooperation of family members. This approach can be used to determine carrier status in familial cases of hemophilia but does not circumvent the problem of uncertain carrier status in sporadic cases that are often due to de novo mutations.

Complete characterization of the factor IX gene and developments in DNA technology during the last decade made it possible to define hemophilia B at the genetic level. Several studies have revealed the high molecular heterogeneity of the defects [2] and the relatively high de novo mutation rate [3]. A number of laboratories have used direct sequencing to analyze the factor IX gene in hemophilia B patients [4, 5].

To screen for factor IX mutations in French hemophilia B patients, and to ensure detection of all female carriers seeking prenatal diagnosis in families at risk, we have used denaturing gradient gel electrophoresis (DGGE), a strategy we have evaluated in the partial analysis of the factor IX gene [6]. In this paper, we applied the technique to full scanning of the gene and to the characterization of 24 novel mutations in patients mostly of French descent.

Samples and Methods

Patient Population and DNA Samples

Forty-nine hemophilia B families were studied. Pedigrees of 3–4 generations, coagulation values, and ethnicity data were sought for each patient. While the majority of the hemophiliacs are French, one is Spanish, one from Tunis, one Belgian, one from Portugal and one from Dakar (Senegal). Family data are presented in table 1. DNA was prepared according to the phenol-chloroform extraction method.

Table 1. Severity of hemophilia B in the families described in this study

Severity[3]	Hemophilia B		
	all (n = 49)	multiple[1] (n = 30)	isolated[2] (n = 19)
Severe	40	25	15
Moderately severe	2	2	0
Mild[4]	7	3	4

1 More than 1 hemophilia B patient in the family.
2 Only 1 hemophilia B patient in the family.
3 Based on the residual factor IX activity (IU/dl): severe < 1; moderately severe 1–5; mild 6–30.
4 Including the Leyden phenotype.

Experimental Design

The 8 exons of the factor IX gene, the surrounding intronic sequences and the promoter and poly(A) regions were analyzed for each patient and potential female carrier. DGGE parameters were determined using the MELT87 and SQHTX programs [7]. These computer algorithms predict the melting behavior of a DNA fragment on the basis of its base composition and sequence. This information was used to select the position of the PCR primers required to generate fragments adapted to DGGE analysis, as well as the range of denaturant concentrations and the run time providing maximum resolution. As the sequence of interest should be contained in the first melting domain of the molecule analyzed, one of the two amplification primers bears an additional 5′-GC-rich oligomer (GC clamp) that introduces a high-temperature melting domain in the PCR product [8].

DNA Amplification

The nucleotide sequences of the amplification primers are shown in table 2. Forty PCR cycles (1 min at 94 °C, 1 min at the relevant annealing temperature and 2 min at 72 °C) were used. Each primer was used at 0.2 μ*M*.

Formation of Heteroduplexes

Heteroduplexes of normal and patient DNAs were performed in the isolated cases when analysis of all the fragments had not clearly revealed electrophoretic anomaly. The amplification products were combined; the mixture was denatured for 10 min at 94 °C and

Table 2. Name and sequences of the primers used for amplification and DGGE analysis of factor IX

Region	Fragment	Primers	Primer sequences (5′–3′)
Exon 1[1]	1	F9E1	GAAATAGTCCAAAGACCCAT
		GCF9E1	(45GC)AAAAGGCAAGCATACTCAATG
Exon 2	2	GCF9E2	(40GC)CATGATGTTTTCTTTTTTGCTA
		F9E2	ATTTTCTATTCTATGCTCTGC
Exon 3	3	GCF9E2	(40GC)CATGATGTTTTCTTTTTTGCTA
		F9E3	CTGATCTTTCTGAGTCCTTT
Exon 4	4	GCF9E4	(40GC)CAGGGGAGGACCGGGCAT
		F9E4	CAGAGGGAAACTTTGAACCA
Exon 5	5	F9E5	AAATGATGCTGTTACTGTCT
		GCF9E5	(40GC)AAGTTTCAGATACAGATTTTC
Exon 6	6	F9E6	TTGCCAATGAGAAATATCAGG
		GCF9E6	(40GC)AATAGCCTCAGTCTCCCACC
Exon 7	7.1	F9E7	TTTCTAGATCAAATGTATTATGCA
		GCF9E7	(30GC)GAGCTAGTGGTGCTGCAGA
	7.2	F9E7i	TCTGTGGAGGCTCTATCGTT
		GCF9E7	(30GC)GAGCTAGTGGTGCTGCAGA
Exon 8	8.1	F9E8	GAAAATCTGTGTATGTGAAAT
		GCF9E8	(40GC)AAAGATGGGAAAGTGATTAG
	8.2	F9E8i	AACCATGACATTGCCCTTCT
		GCF9E8i	(30GC)TTTGCCTTTCATTGCACACT
	8.3	F9E8i2	TCTTAACTGGAATTATTAGC
		GCF9E8	(40GC)AAAGATGGGAAAGTGATTAG
Poly(A)	9	F9PA	AACTAGCATACCCCGAAGTG
		GCF9PA	(30GC)GACTGATTCACATCAATGGA

[1] Exon 1 and promoter region (–92 nt 5′ to the transcription site).

allowed to reanneal for 30 min at 56 °C, thus forming heteroduplexes.

Denaturing Gradient Gel Electrophoresis

The gel apparatus has been described elsewhere [6]. Fifteen microliters of each amplified DNA sample was subjected to electrophoresis at 160 V in a 6.5% polyacrylamide gel containing a linearly increasing denaturant gradient (100% denaturant = 7 *M* urea and 40% formamide). Conditions for DGGE analysis of the factor IX fragments are given in table 3. The sequence of DNA fragments showing a shift in mobility was determined after asymmetric amplification, as described elsewhere [9].

DGGE Multiplex Analysis

Multiplex PCR was performed when conditions were suitable for this analysis. Two fragments, amplified in a single reaction for each DNA sample, were analyzed in a single lane of the denaturing gel.

Factor IX Haplotype Analysis

Polymorphic markers of the factor IX gene, DdeI (intron a), TaqI (intron d), MnlI/Thr-Ala 148 (Malmö) (exon 6) and HhaI (3′ to exon 8) were analyzed in all the families. Except for the Malmö polymorphism, which was detected by DGGE analysis of fragment 6, the analysis was carried out by simple electrophoresis on standard polyacrylamide gel (intron a) or endonu-

Table 3. Amplification and DGGE parameters for analysis of the factor IX gene

Fragment	Size, bp	Annealing temperature, °C	Denaturant range, %	Run time h
1	297	55	10–60	4
2	307	50	10–60	3
3	587	50	10–60	3
4	264	55	10–60	5
5	265	50	10–60	4
6	367	55	10–60	5
7.1	239	55	10–60	4
7.2	160	55	10–60	3
8.1	727	50	10–60	5
8.2	414	55	10–60	3
8.3	237	50	10–60	5
9	280	55	10–60	3

clease digestion of PCR products. We followed the protocol of Winship for the HhaI polymorphism analysis [10].

Results and Discussion

Fragment Design

In the procedure we designed, all the important regions of the factor IX gene can be analyzed by DGGE of 12 gene fragments. Fragment 1 was designed to analyze the near-promoter region (from position −92) and exon 1, as well as the first splice donor site. Fragments 2, 3, 4, 5 and 6 were used to detect mutations in exons 2, 3, 4, 5 and 6, respectively. Exon 7 was amplified in two reactions, with two different and one common primer (table 2). Exon 8, which contains three melting domains, was divided into three fragments, of which two had a common GC-clamped primer (table 2). The poly(A) fragment begins 188 bp 5′ and encompasses 56 bp 3′ to the poly(A) signal. One of the primers of each pair contained a GC clamp.

Factor IX Haplotypes

Haplotype analysis was performed for each hemophiliac and his carrier or noncarrier mother using one extragenic and three intragenic dimorphisms (table 4). The distribution of the different haplotypes in the sample analyzed is shown in table 4. The haplotypes associated with the mutations described are shown for each hemophilia B patient (table 5).

Analysis of the Promoter Region and Exon 1

DGGE analysis of fragment 1 permitted the detection of two known mutations (−6 G-A, 13 A-G) [11–14] (fig. 1) and one new mutation (−20 T-C) in the promoter region, all responsible for a Leyden phenotype. The new mutation, named Marseille (table 5, fig. 1) was found in 1 hemophilia B patient with 9 U/dl of factor IX clotting activity at 3 years of age and 20 U at 8 years. He was the only hemophiliac in the family and the carrier status of his mother (IX:C = 80%) remained undetermined until the identification of the defect. The chromosome assignment showed that the patient had inherited the haplotype associated with the mutation from his grandfather who was not alive at the time of the analysis. It is not clear whether the grandfather was affected by this mild form of the disease or whether the mutation occurred de novo.

Another new mutation, a frameshift at codon −19 (Meaux, 112 Δ1) was also discovered during this analysis (fig. 1). The patient, who is now 11 years old, belongs to a family with several cases of severe hemophilia B. He had severe disease with undetectable factor IX antigen. His mother was informative for the DdeI polymorphism but the grandmother was homozygous for all the markers.

Analysis of Exons Encoding the Propeptide and the Gla Domain

Fragments 2 and 3 were amplified in the same PCR reaction with one common 5′

Table 4. Factor IX markers and haplotypes

a Markers

Marker position	Enzyme	Alleles
Intron a (insertion-deletion of 50 bp)	DdeI	D1 (+50 bp) D2 (−50 bp)
Intron d	TaqI	T1 (−) T2 (+)
Exon 6	MnlI	M1 (−) M2 (+)
8 kb 3′ to exon 8	HhaI	H1 (−) H2 (+)

(−) = Absence of restriction site; (+) = presence of restriction site.

b Haplotypes

Haplotype		Extended haplotype			Frequency[1] %
I	D1 T1 M1	Ia	D1 T1 M1 H1	1111	13.27
		Ib	D1 T1 M1 H2	1112	13.27
II	D2 T1 M1	IIa	D2 T1 M1 H1	2111	20.4
		IIb	D2 T1 M1 H2	2112	25.5
III	D2 T2 M1	IIIa	D2 T2 M1 H1	2211	2.05
		IIIb	D2 T2 M1 H2	2212	2.05
IV	D2 T2 M2	IVa	D2 T2 M2 H1	2221	16.32
		IVb	D2 T2 M2 H2	2222	6.12
V				1122	1.02

[1] Frequency deduced from the French sample of 44 families.

Table 5. List of the hemophilia B patients and the corresponding factor IX mutations identified in this study

Name	Nucleotide change	Amino acid change	Restriction site change	Domain	Factor IX:C, U/dl	Factor IX Ag	Haplotype
Marseille	**−20 T-C**	**none**	–	**promoter**	**7−>20**		**IVb**
Rodez	−6 G-A	none	TaqI (−)	promoter	1−>N		Ia
Poitiers	13 A-G	none	–	promoter	12−>N		IVa
Meaux	**112 Δ1**	**c-19 frameshift**	**RsaI (−)**	**signal peptide**	**<1**	**<1**	**Ib**
Spain	**6325 G-T**	**none (splice)**	–		**<1**		**IIb**
Paris 2	6364 C-T	R-4W	HinfI (−)	propeptide	<1	28	IIb
Caen 2	6364 C-T	R-4W	HinfI (−)	propeptide	<1		IVb
Besançon	6365 G-T	R-4L	HaeIII (−)	propeptide	3		Ia

Table 5 (continued)

Name	Nucleotide change	Amino acid change	Restriction site change	Domain	Factor IX:C, U/dl	Factor IX Ag	Haplotype
Caen	6365 G-T	R-4L	HaeIII (–)	propeptide	2	52	Ia
Tunis	6365 G-A	R-4Q	HaeIII (–)	propeptide	<1		IIa
Autun	**6379 ΔA**	**c2 frameshift**	–	**Gla region**	**<1**	**<1**	**IIb**
Tours	6398-9 Δ2	c8 frameshift	MboII (–)	Gla region	<1	<0.04	IIb
Le Mans	**6424 G-A**	**E17K**	–	**Gla**	**<1**		**IVa**
Pluvigner	6461 G-A	R29Q	BsiI (–)	Gla region	19		IIb
Paris 1	**6494 G-A**	**none (splice)**	**SspI (+)**	–	**<1**		**IVb**
Nantes	6704 T-C	none (splice)	AspI (+)	–			Ia
Lhuis	**10405 T-G**	**C51W**	–	**di-S bond**	**<1**	**<1**	**IVa**
Rouen	**10449 T-C**	**I66T**	**MseI (–)**	**1st EGF**	**30**		**IIb**
Murten	10458 A-G	Y69C	–	1st EGF	<1		IVa
Fort de France	**10468 G-A**	**W72X**	–	**1st EGF**	**<1**		**IIb**
Paris 3	**10471 T-A**	**C73X**	**HphI (+)**	**1st EGF**	**<1**		**IVa**
Paris 5 (double)	10512 A-G	none	–	–	<1		IIa
Chelles	**17691 T-A**	**N92K**	–	**2nd EGF**	**<1**		**Ib**
Amiens	**17691 T-A**	**N92K**	–	**2nd EGF**	**<1**		**Ib**
Paris 5 (double)	**17799 T-C**	**none (splice)**	BspMI (–)	–	**<1**		**IIa**
Dakar	**20374 T-C**	**C132R**	**MaeII (+)**	**di-S bond**	**<1**	**<1**	**IIa**
Brest (double)	**20512 T-C**	**F178L**	**MnlI (+)**	–			
Marseille 2	20518 C-T	R180W	AvaI (–)	cleavage site	<1		Ib
Brest (double)	**20518 C-G**	**R180G**	**AvaI (–)**	**cleavage site**	**<1**	**85**	**IIb**
Hilo, Fr	20519 G-A	R180Q	DdeI (+)	cleavage site	<1	120	ND
Paris 4	**30058 Δ4**	**c202 frameshift**	**NsiI (–)**	**catalytic**	–		**IIb**
Caen 3	**30152InsA**	**c233 frameshift**	**BspMI (–)**	**catalytic**	**<1**	**<1**	**Ib**
Beauvais 1	30800InsA	none	–	–			IVa
Dijon	30864 G-A	R248Q	–	catalytic	5.5		IIIa
Porto	30875 C-T	R252X	TaqI (–)	catalytic	<1		IIb
Besançon 2	**30927 A-G**	**D269G**	**SecI (+)**	**active site Asp**	**<1**		**Ib**
Mantes-la-Jolie	**30957 T-C**	**L279S**	**MnlI (+)**	**catalytic**	**<1**		**IVb**
Fumel	**31005 A-G**	**Y295C**	**BsmI (+)**	**catalytic**	**<1**		**IIa**
Cenon	**31047 G-A**	**G309D**	**MaeIII (+)**	**catalytic**	**<1**		**Ib**
Bruxelles	**31080 C-T**	**A320V**	**AluI (–)**	**catalytic**			**IIa**
Beauvais 2	31118 C-T	R333X	DdeI (+)	catalytic	<1		IIb
Marseille 3	31119 G-A	R333Q	TaqIIB (–)	catalytic	<1		IIb
Poissy	**31200 C-G**	**S360X**	**HinfI (–)**	**catalytic**	**1.5**		**IVa**
Modane	**31200 C-G**	**S360X**	**HinfI (–)**	**catalytic**	**<1**		**IIb**
Bordeaux 2	31200 C-T	S360L	HinfI (–)	catalytic	<1		IVb
Limoges	31218 G-A	G366E	MnlI (+)	catalytic	6		IIb
Chambéry	31220 G-A	G367R	AvaII (–)	catalytic	<1		IVa
Angers	31307 G-A	G396R	–	catalytic	<1	110	IIb
Paris 6	31307 G-A	G396R	–	catalytic	<1		IIa
Chatenay-Malabry	**31329 Δ1**	**c403 frameshift**	**HpaII (–)**	**catalytic**	**<1**		**IIa**
Bordeaux	31352 A-T	K411X	–	catalytic	<1	<1	IIb

Previously undescribed mutations are shown in bold characters.

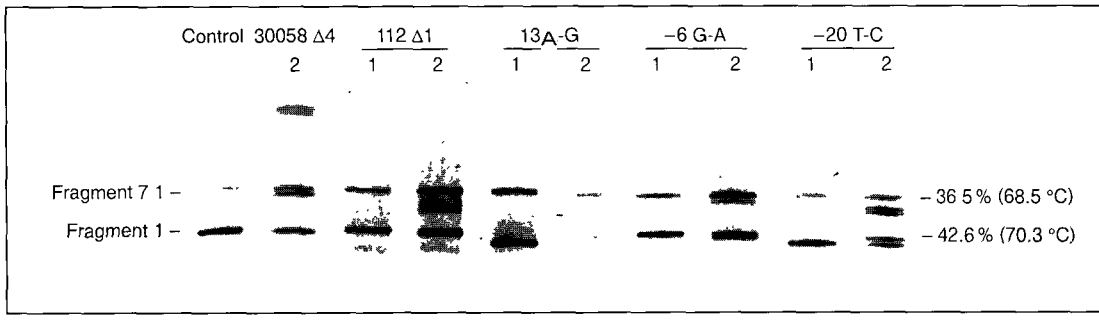


Fig. 1. DGGE analysis of factor IX fragments 1 and 7.1 (table 2). The two fragments were amplified in the same PCR for each DNA sample. For each mutation (except 30058 Δ4), the hemophiliac (lanes 1) and the carrier (lanes 2) are shown. The mutation is indicated for each couple in the corresponding lane. The denaturant concentration corresponding to each fragment and the equivalent temperature (in parentheses) are shown on the right.

primer (GCF9E2) and two 3′ primers (F9E2 and F9E3), and analyzed in the same DGGE gel (not shown). Exon 2 of the factor IX gene encodes the propeptide (codons −17 to −1) and part of the Gla region. Exon 3 encodes the rest of the Gla domain (residues 39–46). DGGE analysis of fragment 2 allowed us to detect 8 mutants (table 5). The 3 mutations of the propeptide have previously been described [3, 15, 16]. The corresponding phenotypes in our patients are given in table 5. The 2 patients bearing the R-4L mutation share the same haplotype, Ia (tables 4, 5).

In the part encoding the Gla domain of the protein, two new molecular defects, codon 2 frameshift, 6379 Δ1 (Autun) and E17K (Le Mans), were found (fig. 2). E17K, changing a γ-glutamic residue of the Gla region to a lysine, caused a severe form of the disease. The two others, a codon 8 frameshift (Tours) and R29Q (Pluvigner) are known mutations [3]. The patient 'Tours' had a severe form of the disease (absence of antigen), whereas 'Pluvigner' had a mild form with 19 U of factor IX:C. Two donor splice defects were also characterized, 6325 G-T (fig. 2) and 6704 T-C; the first, named 'Spain', in a Spanish patient with severe hemophilia B, while the second (Nantes) was found in 2 French sisters of 7 and 14 years who had a hemophilia B phenotype with 3 and 12.5 U of factor IX clotting activity, respectively.

Analysis of Exons Encoding the Two EGF-Like Domains

Five point mutations were identified in exon 4 (codons 47–84) encoding the first EGF-like domain of the factor IX protein. Three were missense mutations of which 2 are new (table 5, fig. 3). C51W (Lhuis) is the first mutation at the cysteine 51 which is involved in 1 of the 3 disulfide bonds contributing to the structure of the protein; the associated phenotype is severe, as would be expected, with very small amounts of factor IX antigen (table 5). In contrast, the second new mutation (I66T-Rouen) is associated with a very mild form of the disease. The third mutation (Y69C-Murten) was first described by Ludwig et al. [unpubl.] and Giannelli et al. [2] as a severe defect. Two novel nonsense defects at codons 72 and 73 (W72X-Fort-de-France and C73X-Paris 3) were identified in patients with a severe factor IX deficiency and undetectable

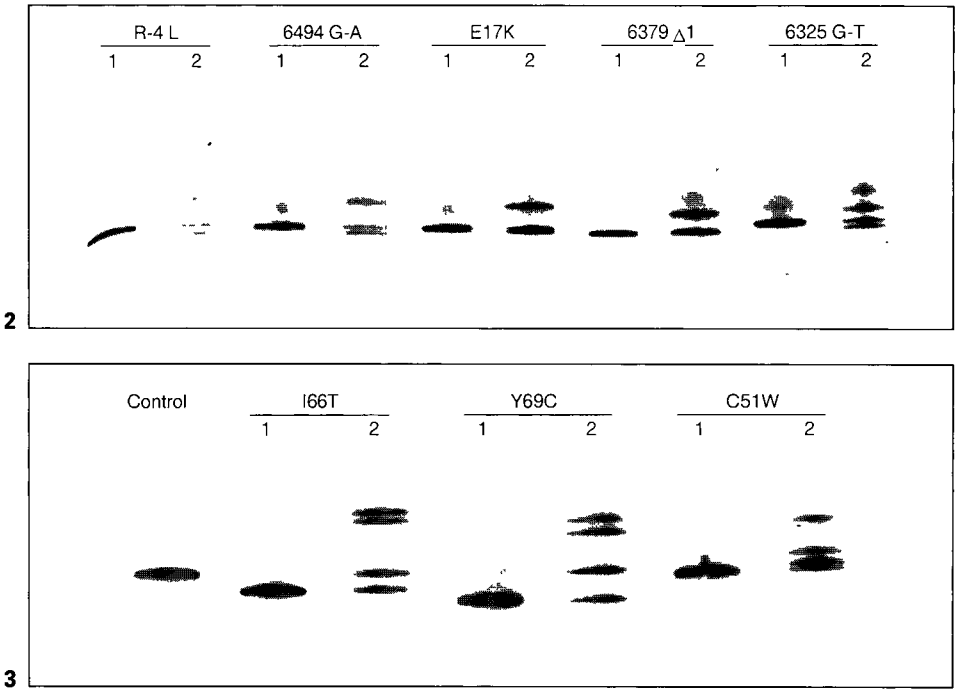


Fig. 2. Patterns of some exon 2 mutations, including 2 splice defects, from the DGGE analysis of fragment 2. For each mutation, the hemophiliac (lane 1) and the carrier (lane 2) are shown.

Fig. 3. DGGE analysis of factor IX fragment 4 (exon 4 and splice junctions). The PCR products were run on a 10–60% gradient gel for 5 h. Lanes 1, hemophiliacs carrying the corresponding mutation; lanes 2, carriers.

factor IX antigen. Only one defect was detected in exon 5, at codon 92 (N92K-Chelles and Amiens) in 2 patients with the same factor IX haplotype, Ib. This amino acid change, from neutral to basic in the 2nd EGF domain, was associated with a severe phenotype in both cases. A new donor splice defect (17799 T-C, Paris 5) was found in 1 patient, and was associated with a severe form of the disease.

Analysis of the Exon Encoding the Activation Peptide

DGGE of fragment 6 was used to detect codon 148 Malmö polymorphism and mutations simultaneously. We detected a number of known mutants at codon 180 (table 5). One of them, factor IX Brest, was coupled to another novel change in the same exon (fig. 4, table 5). Interestingly, only five nucleotides separated the two base changes; the allele R180G, F178L-Brest was observed only in a patient with a severe phenotype. Since the maternal grandfather of the patient was deceased, he could not be analyzed. The patient's mother bore the double substitution whereas the maternal grandmother did not. We deduced that it is likely to be a de novo mutation. The mechanism accounting for this twin substitution is not known, but it is possible that they took place independently. If so, the F178L change may be a variant of factor IX. However, it is unlikely to be a common polymorphism.

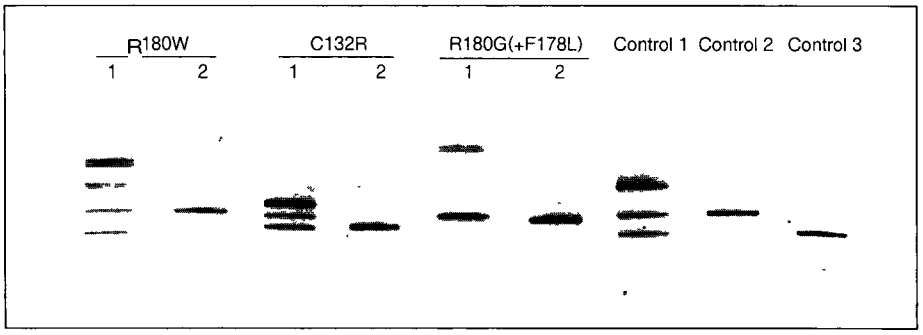


Fig. 4. Patterns of three exon 6 mutants and the Malmö polymorphism (codon 148) from DGGE of factor IX fragment 6. Normal controls heterozygous (control 1) or homozygous (2 and 3) at this site are shown on the right. For each mutation, the carrier is shown in lane 1 and the hemophiliac in lane 2.

Analysis of the Exons Encoding the Serine Protease Domain

Exploration of the catalytic domain of factor IX, encoded by exons 7 and 8, was achieved in five steps, given the size of exon 8 and the melting maps of the two exons. Exon 7 was analyzed using three amplification primers, whereas five primers were used for PCR-DGGE of exon 8, as shown in table 2. Among the 18 mutations observed in this domain in our patient cohort, 9 were new. Two novel frameshift mutations were observed in exon 7 (table 5). One of the cases (Paris 4) (fig. 1) was particularly interesting and illustrates the need for direct methods in this type of disease. Hemophilia B was strongly suspected in 2 sisters and their mother on the basis of coagulation data but there was no family history of hemophilia. Unfortunately, all 3 members were uninformative for the markers tested. The identification of the mutation made it easy to confirm the status of the other relatives and to propose early prenatal diagnosis to those at risk.

Seven undescribed mutations (5 missense, 1 nonsense and 1 frameshift) were found in exon 8. The remaining mutations have been reported elsewhere [4, 17–20] (table 5). One (Dijon, R248Q) (fig. 5), described for the first time by Chen et al. [21], was associated with mild disease, in agreement with a recent functional study of this mutant [22]. The latter authors found that the mutant protein had 41% of the activity of normal factor IX. In addition, the mutation was associated with an unusual haplotype, IIIa (see table 4), found once in our sample.

Different novel defects have been identified in exon 8 during this study. A number of them are shown in figures 5–7. The mutant Bordeaux (K411X) is the same as that described previously [6]. Patients Angers and Paris 6 (G396R) had different haplotypes at the HhaI allele (IIb and IIa, respectively). HhaI is not in association with the other factor IX RFLPs [10, 23]. This result thus indicates independent origins of these mutations.

Finally, the patients Modane, Beauvais, Besançon 2 and Limoges were characterized after heteroduplexing with normal controls, as described in Methods, because there was no evidence of the carriership of their mothers, and no clear shift was detected upon DGGE of the different fragments. Indeed, the corresponding mutations involved nucleotide changes that are difficult to distinguish in

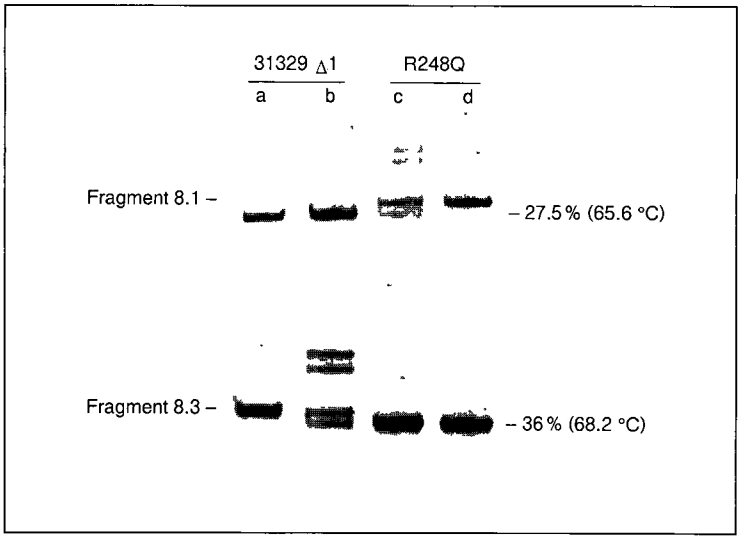
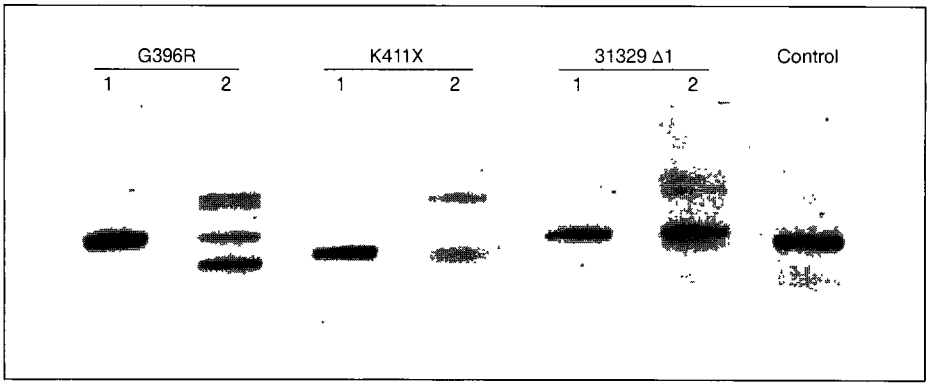


Fig. 5. Simultaneous DGGE analysis after co-amplification of factor IX exon 8 fragments 8.1 and 8.3. Fragment 8.1 includes the 5′ part of exon 8. The percentage of denaturants corresponding to each fragment and the equivalent temperature (in parentheses) are shown on the right. Lane a: the patient hemizygous for the mutation 31329Δ1, detected in fragment 8.3; lane b: his carrier mother, lanes c and d: the mother and the hemophiliac carrying the mutation R248Q, respectively.



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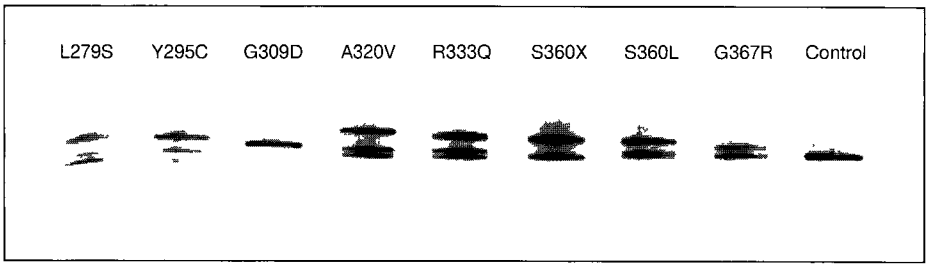

7

Fig. 6. DGGE analysis of fragment 8.3 alone showing different mutations in the 3′ part of exon 8. Patients Angers (G396R), Bordeaux (K411X), Chatenay-Malabry (c403 frameshift, 31329Δ1) are shown in lanes 1; the carriers of these mutations are shown in lanes 2.

Fig. 7. Analysis of the major part of exon 8 by electrophoresis of fragment 8.2. Several mutations are shown only in heterozygote carriers and indicated in each lane, except for the mutation G309D (Cenon), which is shown in the hemophiliac.

homoduplexes, and the mutation would therefore escape detection by DGGE. In the case Beauvais, the mother was a carrier of the neutral change Beauvais 1 (table 5) and the hemophiliac son bore the de novo occurring nonsense mutation, R333X (Beauvais 2). Therefore, Beauvais was not a double mutation, the two changes occurring on different X chromosomes.

Analysis of the Poly(A) Signal Sequence

Fragment 9 was designed for analysis of the poly(A) signal (the consensus AATAAA) with a limited flanking sequence. This analysis was only performed once the scanning of the whole gene and heteroduplexing were achieved without positive result. We have not observed any mutant of this region in our sample.

Conclusion

By screening of DNA mutations by means of DGGE, we established the molecular basis of hemophilia B in 49 patients with severe, moderate or mild forms, finding 24 novel, causative mutations. Identification of carrier females is crucial for genetic counselling in hemophilia B; the problem is that hematological data are equivocal unless the factor IX level is very low or when, in members of families segregating for CRM+ mutations, coagulant activity is much more reduced than the factor IX antigen level. Only direct identification of factor IX mutations can solve this problem. We have opted for DGGE of PCR products as a simple, rapid, exhaustive, nontoxic and nonradioactive screening method. Furthermore, it allows rapid DNA sequencing to be carried out once mutations have been detected. Our strategy, involving analysis of the whole factor IX coding sequence and flanking regions as well as the near-promoter, requires DGGE of 11 fragments. The procedure proved highly efficient: several new defects were identified in addition to a number of known mutations. Our results further document the high molecular heterogeneity of hemophilia B and define a part of the factor IX mutations found in the French population. An important consequence of this direct approach is that we were able to solve the sporadic cases studied, as well as the cases that lacked informative DNA markers. The method made it possible to determine without ambiguity the carrier status of women seeking prenatal diagnosis, thus improving genetic counselling. In this way, early prenatal diagnosis is only carried out for women at risk.

Finally, the possibility of multiplex analysis of different fragments of the factor IX gene makes this direct analysis strategy faster and more efficient, and makes DGGE the method of choice for screening and diagnostic purposes. Hence, it is unlikely that in the unique unsolved case of our sample, the mutation had escaped detection. However, it is possible that the defect occurred in regions not explored by this strategy, i.e. the intronic sequences, the up-promoter or regulatory extragenic regions.

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