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Parental Origin of Factor IX Gene Mutations, and Their Distribution in the Gene

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Summary

Genomic amplification followed by direct sequencing enabled us to establish the causative mutation in 67 unrelated hemophilia B patients of predominantly German origin. With the detection of the mutation, extensive pedigree analysis has become feasible. We therefore anticipated that determination of the origin of mutation could be achieved in a comparatively great number of families. Although these investigations often were restricted by the availability of blood samples from the maternal grandparents or great-grandparents, we were able to prove a de novo mutation in 9 of 20 families with sporadic hemophilia B and in 3 of 20 families with a history of the disease. This could be achieved with the aid of RFLP analysis and, in one case, where the mutation is still unknown, with the aid of biochemical and immunological factor IX assays. Since the maternal grandfather was deceased in two of these families, the germ line of origin could not be determined precisely. In the remaining families, the female and male germ lines turned out to be the origin of mutation in six and four cases, respectively, and an effect of paternal age on the mutations observed could not be excluded. Furthermore, our data indicate that the hemophilia B gene pool is mainly renewed by variable mutations.

Introduction

The development of methods for the amplification of genomic DNA (Saiki et al. 1988) and for direct sequencing of amplified PCR products (Wong et al. 1987) has greatly facilitated the detection of mutations in various diseases and, as a result, has improved carrier and prenatal diagnosis. Furthermore, it has become feasible to rapidly prove de novo mutations and to determine the germ line of origin. On the basis of these data, reliable estimates of mutation rates, the sex ratio of mutation, and paternal age effect could be obtained.

Apart from abnormal chromosome numbers and structural chromosome aberrations, a direct calculation of the mutation rate (μ = [number of sporadic cases with a certain anomaly]/$2x$ [x = number of individuals examined] [Vogel and Motulsky 1986]) has thus far been confined to dominant gene mutations with complete penetrance, since the detection of heterozygotes in recessive X-linked or recessive autosomal traits has been difficult or impossible. More difficulty arises in establishing the proportion of sporadic cases and in determining whether a case is truly sporadic. Particularly in mild and moderate forms of a disease, pedigrees often show a single affected member of the family and yet have familial "founder effect" mutations.

Except for autosomal recessive traits, Haldane's (1935) indirect method could instead be used. Regarding X-linked recessive hemophilia, Haldane initially suggested that the reduced fitness of the patients would cause the disease to disappear if it were not balanced by mutations. Since males have one-third of the X chromosomes in the population, the mean mutation rate (μ) per X chromosome is equal to one-third of the proportion of hemophilic males in the population (x), modified by a reproductive fitness factor (f): $\mu = 1/3(1-f)x$.

However, in the application of this method prob-

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lems arise that would lead to different mutation rate estimates. This is dependent on the estimation of the effective fertility and on an often unknown value of the sex ratio of mutation.

With regard to hemophilia, the approximate population equilibrium, which depends on the introduction of new mutations and on the loss of hemophilic genes because of the patients' reduced fitness, is shifting (Rosendaal and Briet 1990). On the basis of a more effective therapy, the observed increasing prevalence of hemophilia decreases the turnover in the gene pool. Haldane's formula (see above) suggests that for a recessive X-linked lethal disorder the proportion of sporadic cases should be one-third if mutation rates are equal in both sexes. A value of one-sixth has been estimated in case of hemophilia B (Ferrari and Rizza 1986), and the apparent discrepancy of sporadic cases has been explained by significant fitness of affected males and/or by an increased ratio of male-to-female mutation rates.

Recessive X-linked hemophilia B that is caused by deficiency or functional abnormality of blood-clotting factor IX now for the first time offers an advantageous system to obtain a reliable order of magnitude of the mutation rate by the application of the direct calculation method. On the basis of sequence data obtained from 60 hemophilic genes, mutation rates per base pair per generation have been calculated for transitions, transversions, and deletions in the factor IX gene (Koeberl et al. 1990*a*). Nevertheless, although the causative mutation in more than 400 patients afflicted with hemophilia B has been detected (for reviews, see Furie and Furie 1990; Giannelli et al. 1991), to our knowledge, only in nine cases has the origin of the mutation been proved (Vidaud et al. 1986, 1989; Tanimoto et al. 1988; Bottema et al. 1989*a*, 1990*a*; Koeberl et al. 1990*b*; Montandon et al. 1990; Wang et al. 1990; Taylor et al. 1991). Besides the detection of a somatic mosaic, the female and male germ lines have been the origin of mutation in four cases each.

We have investigated factor IX gene regions of functional significance in a total of 73 unrelated hemophiliacs, and the causative mutation could be delineated in 67 of these patients (Ludwig et al. 1989, 1991; Giannelli et al. 1991). In an attempt to determine the origin of the observed mutations, extensive pedigree analysis was carried out in 20 families with sporadic cases and in another 20 families with more than one affected member. A de novo mutation could be proved in 12 families, and the germ line of origin was determined in 10 cases.

Material and Methods

Blood samples were obtained from 73 hemophilic patients of predominantly German origin and from relevant family members. Coagulation values were obtained according to a method described elsewhere (Ludwig et al. 1989). Genomic DNA was extracted from white blood cells by the use of standard techniques, and RFLP analysis was performed according to a method described elsewhere (Ludwig et al. 1989). The *Dde*I RFLP was also determined, according to the protocol of Hanauer and de la Salle (1990), and the Malmö polymorphism (thr or ala at amino acid 148), (McGraw et al. 1985; Winship and Brownlee 1986) was established in the course of direct sequencing.

PCR was carried out according to the method of Saiki et al. (1988) by using a DNA thermal cycler and the GeneAmp™-kit (Perkin Elmer—Cetus) as specified by the manufacturer. The appropriate oligonucleotide primers and 0.5 μg of genomic DNA were used to amplify all factor IX coding regions, the polyadenylation signal region, and the 5′ region containing both the transcription site assigned by Anson et al. (1984) and Salier et al. (1990), as follows: cycles of a 30-s denaturation at 94°C, a 45-s annealing at 52°C–58°C (depending on the primers used), and a 180-s elongation at 72°C were repeated 35 times, with the elongation time being prolonged for 10 s in each cycle. Five microliters of the reaction volume were assayed on a 2% NuSieveGTG (Biozym) agarose gel, and the remainder amplification product was purified by spin dialysis in a Centricon-30 microconcentrator (Amicon GmbH). Preferential single-strand amplification in a second amplification step was omitted, and about one-tenth of the double-stranded purified PCR product was subjected to direct nucleotide sequencing. The dideoxy chain-termination method (Sanger et al. 1977) was carried out by the use of the Sequenase™ sequencing kit (USB Corp.) and [γ-^{32}P]ATP (Amersham) 5′-labeled sequencing (S) primer.

The amplification (A) and (S) primers were as follows (the corresponding nucleotides of the factor IX gene [Yoshitake et al. 1985] are indicated in parentheses): promoter (P)/exon (E) I5′A (−430 to −411), P/EI3′A (141 to 121), PS1 (−240 to −222), PS2 (−85 to −65), EIS (−9 to 10), EII/III5′A (6244 to 6265), EII/III3′A (6741 to 6720), EIIS (6292 to 6312), EIIIS (6627 to 6647), EIV5′A (10315 to 10333), EIV3′A (10548 to 10529), EIVS (10357 to 10375), EV5′A (17584 to 17603), EV3′A (17849 to 17829), EVS (17636–17655), EVI5′A (20299 to 20319), EVI3′A

Table 1

Familial History and Severity of Hemophilia B in 73 Unrelated Patients

Clinical Severity (factor IX coagulant value)	No. (%) of Sporadic Cases	No. (%) of Nonsporadic Cases	Total (%)
Severe (<1)	37 (82.2)	15 (53.6)	52 (71.2)
Moderate (1–5)	4 (8.9)	9 (32.1)	13 (17.8)
Mild (>5)	4 (8.9)	2 (7.1)	6 (8.2)
Leyden phenotype[a]	—	2 (7.1)	2 (2.7)
Total	45	28	73

[a] Factor IX coagulant value varies with age and rises after puberty.

(20632 to 20615), EVIS1 (20322 to 20343), EVIS2 (20431 to 20449), EVII5′A (29962 to 29980), EVII3′A (30192–30174), EVIIS (30000 to 30020), EVIII5′A (30750 to 30770), EVIII3′A (31419 to 31399), EVIIIS1 (30777 to 30799), EVIIIS2 (30948 to 30966), EVIIIS3 (31088 to 31106), EVIIIS4 (31226 to 31244), polyA 5′A (32573 to 32592), poly A3′A (33488 to 33470), and poly AS (32638 to 32656).

Results

Direct sequencing of eight PCR products constituting the factor IX regions of functional significance (see Material and Methods; also see Ludwig et al. 1991) enabled us to establish the causative mutation in 67 of 73 unrelated hemophiliacs (Ludwig et al. 1989; Giannelli et al. 1991). Six mutations are still unknown and remain to be elucidated.

Table 2

Origin and Type of Mutation in 12 Families Afflicted with Hemophilia B

Factor IX Type	Coagulant (%)	Antigen[a] (%)	Nucleotide Position,[b] Mutation	Amino Acid[c] Change	Germ Line of Origin	Age at Conception (years)	Sporadic
Riegelsberg[d]	<1	<1	37, G→A; and 31,084–31,090, del AGTTCTT	Frameshift	Mother	30	+
Bonn 2	<1	<1	6,402–6,406, del TGTTC	Frameshift	Mother	22	+
Heesen	<1	ND	6,427, T→C	18, C→R	Mother	25	+
Rheidt	2	32	6,449, T→C	25, F→S	Maternal grandfather/ Maternal grandmother[e]	27/24	–
Würzburg	<1	ND	17,763, ins C	Frameshift	Maternal grandfather/ Maternal grandmother[e]	27/21	–
Nörtingen	<1	ND	17,798, G→T	Donor splice	Maternal grandmother	27	+
Dernbach	<1	69	20,518, C→T	180, R→W	Maternal grandfather	43	+
Köln	2	60	31,119, G→A	333, R→Q	Maternal grandfather	46	–
Düsseldorf	4	86	31,119, G→A	333, R→Q	Mother	20	+
Offenbach	<1	ND	31,166(67), del T	Frameshift	Maternal grandmother	29	+
Hannover[f]	<1	<1	del 9,900–19,218	del exons d and e	Maternal grandfather	23	+
Adenau[g]	<1	<1	?	?	Maternal grandfather	32	+

[a] ND = not done.

[b] Numbering is according to Yoshitake et al. (1985).

[c] Numbering is according to Anson et al. (1984).

[d] Double mutant: G-to-A (nt 37) is designated by base pair only, since amino acid −44 is probably not translated (Pang et al. 1990); only the origin of the deletion could be proved.

[e] The maternal grandmother was found to be not a carrier; since the normal maternal grandfather was deceased, the germ line of origin could not be established.

[f] Previously reported by Ludwig et al. (1989); precise characterization of the deletion junction.

[g] Origin of the mutation was predicted with the aid of factor IX coagulation and antigen values.

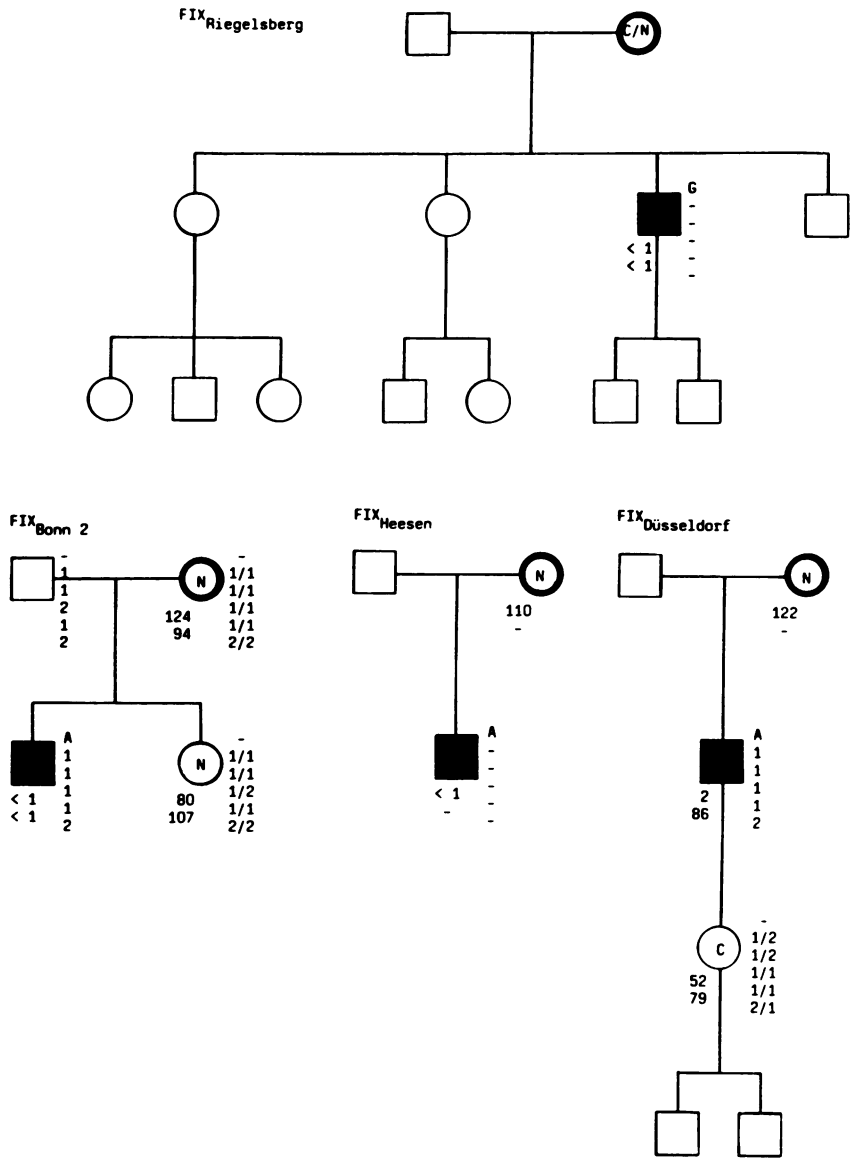

Figure 1 Abbreviated pedigrees of four families in which a de novo mutation arose in the germ line of the mother. Factor IX coagulation values (FIX:C and FIX:Ag) and haplotypes (Malmö allele, *Xmn*I, *Taq*I, *Dde*I, *Bam*HI, and *Msp*I) in succession are listed below and next to the subject symbols, respectively; a dash (–) denotes that the value was not determined. C/N in family hemophilia $B_{Riegelsberg}$ indicates that the mother carries only the G-to-A transition observed in the patient but not the deletion. © = Carrier by sequence analysis; ⊘ = noncarrier by sequence analysis; □ and ○ = germ line of origin.

On the basis of these findings, from a total of 73 families (see table 1), extensive pedigree analysis was carried out in 20 families with sporadic disease and in 20 families with a prior history of hemophilia B. Sixty females at risk were diagnosed by direct carrier testing (data not shown), and in 12 families the occurrence of a de novo mutation could be proved; the germ line of origin was established in 10 cases (figs. 1–3 and table 2).

In four families with sporadic disease (fig. 1), sequencing of the appropriate PCR product obtained from the mother's blood revealed only the normal factor IX sequence. This indicates that the deletions found in patients with hemophilia $B_{Riegelsberg}$ and he-

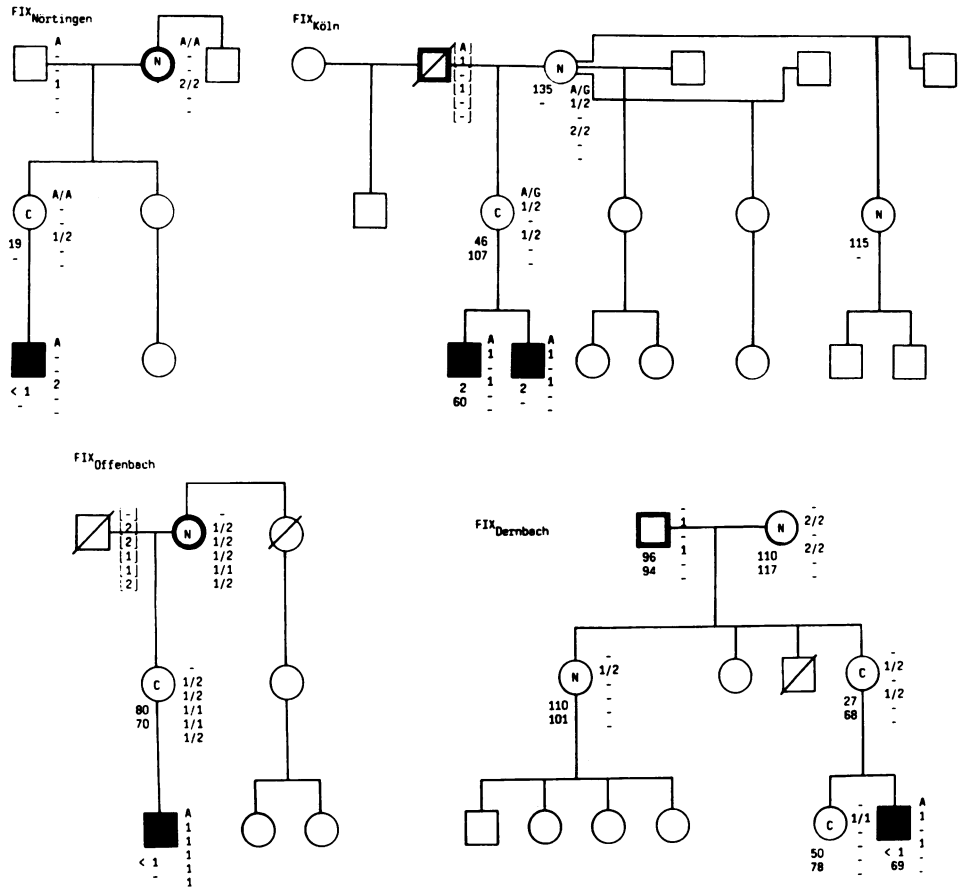

Figure 2 Pedigrees of four families in which the origin of the de novo mutation has been established with the aid of haplotype analysis. Symbols are as in fig. 1.

mophilia $B_{Bonn\ 2}$, as well as the transitions observed in patients with hemophilia B_{Heesen} and hemophilia $B_{Düsseldorf}$, in each case arose in the germ line of the mother. The second mutation (G to A at nucleotide 37) found in the double mutant factor $IX_{Riegelsberg}$ gene is likely to be a normal variant, and its origin could not be proved, since the maternal grandparents were deceased. In all four cases the mother was not older than 30 years (30, 22, 25, and 20 years, respectively) at the time of conception of her hemophilic son.

The germ line of the maternal grandmother has been determined to be the origin of both the transversion found in factor $IX_{Nörtingen}$ and the deletion that could be observed in factor $IX_{Offenbach}$. In both sporadic cases, the mother was found to be a carrier, and haplotype analysis indicated that the affected son has inherited the hemophilic allele from his normal maternal grandmother (see fig. 2). As in the case of the four mothers mentioned above, the maternal grandmother was not older than 30 years (27 and 29 years, respectively) at the time of conception of her carrier daughters.

With the aid of RFLP analysis the transitions observed in factor $IX_{Dernbach}$ and factor $IX_{Köln}$ were found to have arisen in the sperm of the maternal grandfather (fig. 2). In both cases, an increased age (43 and 46 years, respectively) at the time of conception of the carrier daughter could be observed.

The deletion in factor $IX_{Hannover}$ (Ludwig et al. 1989), which is now documented precisely (table 2), must also have originated in the maternal grandfather. Haplotype analysis showed that the mother is lacking at least two polymorphisms she should have inherited from the maternal grandfather (see fig. 3).

There is also strong evidence that the mutation in factor IX_{Adenau} must have originated in the maternal grandfather. Sequencing of all regions of likely func-

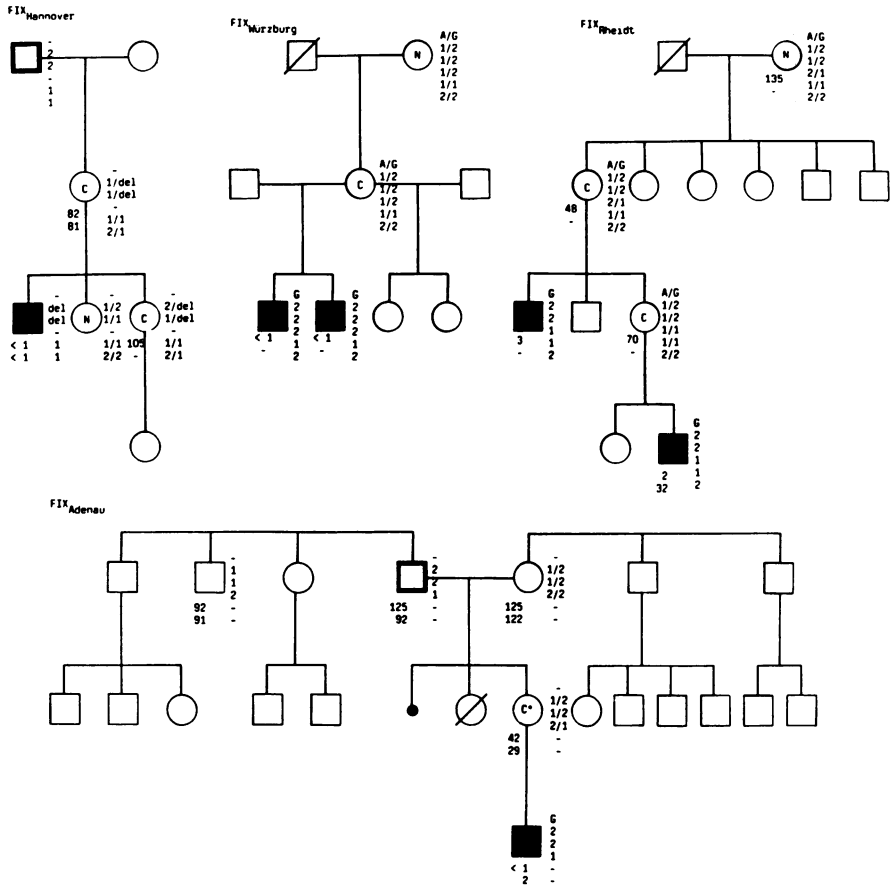

Figure 3 Pedigrees of families with hemophilia $B_{Hannover}$, hemophilia B_{Adenau}, hemophilia $B_{Würzburg}$, and hemophilia B_{Rheidt}. In the former two families the origin of the mutation could be proved by haplotype analysis and with the aid of coagulation values, respectively. The germ line of origin could not be established in the latter two families. Symbols are as in fig. 1. ⓒ = Carrier by factor IX coagulation values.

tional significance revealed no deviation from normal, and hemophilia B_{Adenau} might therefore be due to an additional intronic splice site. Nevertheless, coagulation values obtained from the mother (FIX:C 42%) indicate that she is a carrier of the mutant gene that has been transmitted to her by the healthy father (see fig. 3). With the exception of factor $IX_{Köln}$, where the carrier mother gave birth to two hemophilic sons, the de novo mutations observed in the male germ line have all been proved in families afflicted with sporadic hemophilia B.

In two further families with a history of the disease, only the generation in which the mutation originated could be established. The maternal grandmother was found to be not a carrier of the mutant gene in either factor IX_{Rheidt} or factor $IX_{Würzburg}$. However, the healthy maternal grandfather was deceased, and haplotype analysis gave no definite clue of the paternal alleles transmitted to the carrier daughter (see fig. 3). Hence the germ line of origin could not be established. An increased grandpaternal age at the time of conception has not been observed in either of the families.

Seven of the reported mutations newly introduced into the factor IX gene have not been reported by other investigators thus far, thereby suggesting that the hemophilia B gene pool is mainly renewed by variable mutations. The de novo occurrence of recurrent mutations has been observed at arginine residues 180 and 333 and at cysteine residue 18. On the other hand, none of our patients showed a mutation in the frequently affected codons gly60, arg145, arg252, and ile397 (Giannelli et al. 1991). The pattern of the de

novo mutations observed (five transitions, one transversion, one insertion, and four deletions) is in contrast to the frequencies calculated by Koeberl et al. (1990*a*), since an unexpectedly high proportion of insertion/deletion events has been observed in our comparatively small sample.

Discussion

Direct sequencing of amplified genomic DNA enabled us to prove 12 de novo mutations in an unselected sample of 40 families afflicted with hemophilia B. Among these cases an unexpectedly high proportion of deletion/insertion events (42%) was observed. If the five de novo deletions/insertions determined thus far (Vidaud et al. 1986, 1989; Tanimoto et al. 1988; Bottema et al. 1989*a*, 1990*a*) were added, this value would rise to 48%. In contrast, a total of 72 deletions and insertions (17.1%) have been observed in 420 hemophilia B patients investigated so far (Furie and Furie 1990; Giannelli et al. 1991). These differences in the pattern of mutation might be explained by biased ascertainment. The sample in the present study is clearly biased toward severe disease (table 1), as the proportions of mild, moderate, and severe disease are not population based (see Bottema et al. 1990*a*). In the case of hemophilia A it has been estimated that about 5% (Tuddenham 1989) of the molecular defects leading to the disease are gross deletions. However, the screening was skewed toward these mutations. As in hemophilia B, this value may increase because small deletion/insertion events are undetectable by the Southern blotting technique.

Nevertheless, the high proportion of these rearrangements newly introduced into the factor IX gene suggests that these types of mutation, in general causing the severe form of the disease, might be responsible for hemophilia at a higher frequency than has been observed today. This assumption depends on (1) the excess mortality of severely affected patients in the past and (2) the fact that hemophiliacs reproduce less frequently than do men in the general population (Rosendaal and Briet 1990). This would mainly lead to a decrease of those hemophilic genes which are responsible for the severe form of the disease in the gene pool. In fact, the proportion of severely afflicted patients

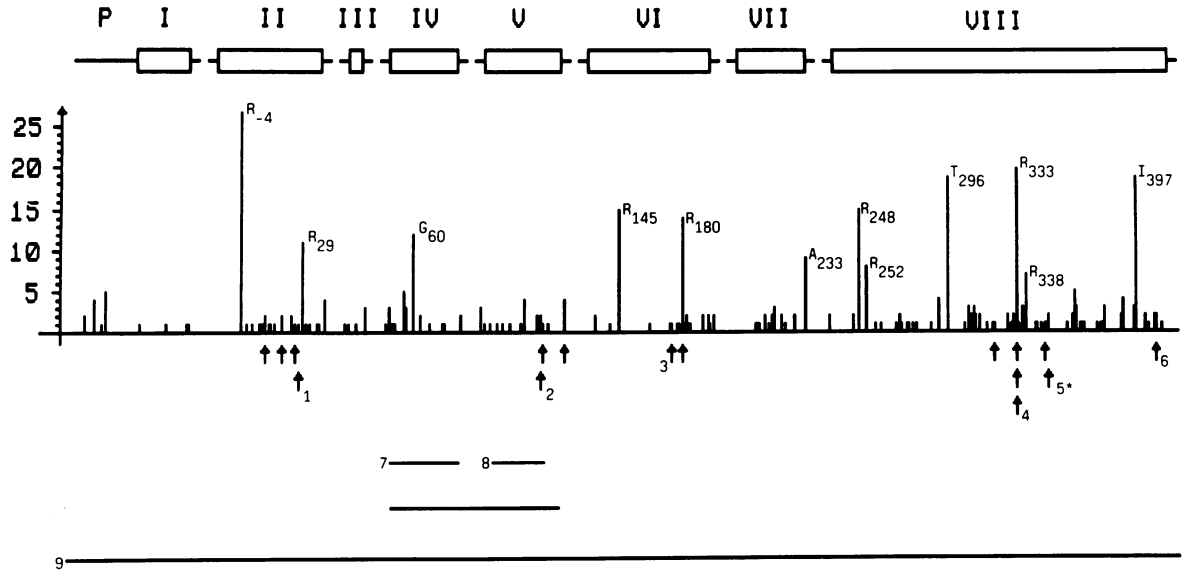

Figure 4 Line diagram of the factor IX gene, showing the putative promoter (P) and exons (solid bars) I–VIII along with their adjacent acceptor and donor splice sites. The frequency and location of point mutations and short additions/deletions observed thus far (Giannelli et al. 1991) are indicated, with each bar representing an amino acid. Numbering of frequently affected residues is according to Anson et al. (1984). De novo mutations, where the germ line of origin has been determined, are indicated by arrows (point mutations) and by vertical bars (gross gene deletions and insertions), respectively. Numbers 1–9 signify the de novo mutations reported by Wang et al. (1990), Montandon et al. (1990), Bottema et al. (1990*a*), Koeberl et al. (1990*b*), Taylor et al. (1991), Bottema et al. (1989*a*), Vidaud et al. (1986), Vidaud et al. (1989), and Tanimoto et al. (1988), respectively. The somatic mosaic identified by Taylor et al. (1991) is indicated by an asterisk.

(53.6%) in families with a prior history of hemophilia B is significantly smaller than their number (82.2%) in families with sporadic disease (see table 1).

As illustrated in figure 4, recurrent mutations have been observed at various codons in the factor IX gene (for review, see Giannelli et al. 1991), and four transitions at arginine codons 180 and 333 (Koeberl et al. 1990*b*; present study) could be proved to be de novo mutations. This indicates that these loci are genuine sites of frequent mutations, whereas other mutations often observed might be due to a common ancestor of these patients. The ile-to-thr substitution at codon 397, for example, has been detected in 19 hemophilic genes thus far (Giannelli et al. 1991), and a "founder" effect has been proposed to explain these occurrences (Bottema et al. 1989*b*, 1990*b*; Thompson et al. 1990). The view that codon 397 is not a hot spot of mutation is further supported by our findings, since none of the 73 patients investigated had this mutation.

In addition, none of our patients showed one of the frequently observed mutations affecting the arginine codons at position 145 and 252 and the glycine codon at position 60 (see fig. 4). Therefore, most of these occurrences might also be due to a "founder" effect. Primarily, the recurrent mutations at residues 60 and 145 might have occurred in a common ancestor of these families, particularly, since the mutations observed thus far (Giannelli et al. 1991) lead to moderate or mild hemophilia B.

The ratio of patients who are sons of normal mothers not carrying the hemophilic gene can be estimated according to the formula of Haldane (1935). Let μ and ν be the mutation frequencies in the female and male germ lines, respectively, and let f be the average fertility of patients relative to the population average; the probability (m) that the mother of a patient is homozygous normal is $(1-f)\mu/(2\mu+\nu)$. Given a mutation rate of 2.5×10^{-6} (Vogel and Motulsky 1986), an equal sex mutation rate, and an effective fertility of hemophilics of .7 (Ikkala 1960; Ferrari and Rizza 1986; Istvan et al. 1990) m becomes .1. Hence 10% of all hemophilia B patients should be sons of normal homozygous mothers. This estimate is in agreement with our observation: in the present study direct sequencing revealed noncarriership of the mother of 4 (10%) of 40 families. If one proceeds on the assumption that m (see equation above) is derived from reliable values (μ,f), the results are in accordance with an equal mutation rate in both sexes. However, a larger sample has to be investigated to obtain reliable data.

Comprehensive theoretical reflections on mutation rates and parental age effect have been presented by Vogel and Motulsky (1986). The increase of the mutation rate only with paternal age is explained as being due to continuous dividing of the spermatogonia, in contrast to the very few cell divisions in the female cell line. This would consequently lead to a higher mutation rate in the male germ line compared with the female germ line (model 2 in Vogel and Motulsky 1986). Opposite observations might be obtained if mutations were to occur after cessation of divisions (model 4). On the other hand, mutations depending on time only would lead to a linear increase of mutations with age, thereby showing no sex difference (model 1). Furthermore, mutations in mature germ cells (model 5) or during a certain time before puberty (model 3) would show neither an increase with age nor sex difference.

Our observations indicate that there might be an effect of paternal age on mutations observed in the factor IX gene. The paternal origin of the mutation could be proved in factor $IX_{Köln}$ and factor $IX_{Dernbach}$, among others. In these two families the maternal grandfather was 43 or 36 years old at the time of conception of his carrier daughter. Similar observations have been made by Barrai et al. (1985). No effect of maternal age on mutations could be observed in our study (table 2).

On the other hand, at present a total of 18 germ lines of origin have been determined for hemophilia B, and the female germ line turned out to be the origin of mutation in 56% of these cases. This nearly equal sex ratio of mutation rates seems to be inconsistent with the mutation mechanism proposed in model 2, thereby suggesting that other mechanisms (models 1 and 3–5) might also account for the incidence of mutations observed in the factor IX gene.

No sex difference in the mutation rate has also been observed in the case of Duchenne muscular dystrophy (Yasuda and Kondo 1980). Furthermore, no effect of parental age on the rate of mutation has been demonstrated for this disease (Davie and Emery 1979; Yasuda and Kondo 1982). In the case of hemophila A, contradictory indirect measurements have been published. A maximum-likelihood estimate of the male-to-female mutation ratio of 9.6 has been obtained by Winter et al. (1983), whereas no mutation-ratio difference between sperm and eggs could be demonstrated by Barrai et al. (1985). Besides a heterogeneity of the sex ratio of mutation rates at different loci on

the X chromosome, these findings might also suggest an involvement, in the formation of mutations, of probably all mechanisms mentioned above.

Because of the scarcity of data we will make no attempt to directly estimate the mutation rate in the factor IX gene. Nevertheless, this might be achieved in the near future, since the causative mutation has been delineated in 400 patients so far, thereby facilitating extensive pedigree analysis and the search for de novo mutations. PCR and direct sequencing will also be the method of choice in various other diseases, to obtain reliable data for a direct estimate of mutation rate and to finally address the questions of whether there is (*a*) an effect of parental age on mutation and (*b*) an unequal sex ratio of mutation.

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