Analysis of large structural changes of the factor VIII gene, involving intron 1 and 22, in severe hemophilia A

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Background and Objectives. Hemophilia A (HA), the deficiency of coagulation factor VIII (FVIII), is the most common, sex-linked inherited bleeding disorder. The disease is caused by FVIII gene intron 22 inversion in approximately 50% of the patients, and by intron 1 inversion in 5% of the patients with severe HA. Both inversions occur as a result of intrachromosomal recombination between homologous regions, in intron 1 or 22, and their extragenic copy located telomeric to the FVIII gene. The goal of the present study was to analyze the presence of large structural changes in the FVIII gene in patients with severe hemophilia A.

Design and Methods. We studied 104 unrelated, severe HA-patients or obligate carriers for the presence of intron 22 and intron 1 inversions by Southern blotting, long-distance polymerase chain reaction (PCR), and simple PCR.

Results. We found altered intron 22 restriction profiles by Southern analyses in 58 cases: 43 type 1, 11 type 2 inversions and 4 unusual patterns. Upon further examination of the last 4 cases, large deletions involving intron 22 were demonstrated in two cases. In the remaining two patients extra homologous regions were detected by Southern analysis, and long-distance PCR showed the presence of unaltered intra- and extragenic copies together with one inversion-affected copy, suggesting that an additional intronic fragment participated in the inversion process and was inserted in the genome. During screening for intron 1 inversion among 43 patients, who were intron 22 inversion negative, we identified only wild type individuals.

Interpretation and Conclusions. The relatively large proportion of unusual patterns further supports the observation that the structure of FVIII intron 22 represents a hot spot for large gene rearrangements with various mechanisms, while intron 1 inversion seems to be not common in Hungary.

Key words: hemophilia A, factor VIII, intron 22 inversion, intron 1 inversion, long-distance PCR.

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Hemophilia A (HA), the deficiency of the coagulation factor VIII (FVIII), is the most common, sex-linked inherited bleeding disorder. The disease is caused by a wide variety of heterogeneous and infrequent mutations in the FVIII gene. The only identified mutation hot spot of the FVIII gene is its largest intron (intron 22), with 40 to 50% of severe HA cases arising from intron 22 inversions. Recently, an inversion breaking intron 1 was reported to occur with a frequency of 5% among British patients with severe HA, and this may represent the second most common mutation.

The intron 22 of the FVIII gene contains a 9.5 kb region, which is present outside of the gene, near the telomere of the X chromosome in two additional copies. The high degree of identity and the opposite direction of the extragenic copies, compared to the intragenic homologous region, promote intrachromosomal recombination during male gametogenesis, and contribute to the high rate of occurrence of chromosomal rearrangements in this region.[1] Depending upon which extragenic homologous region is involved, two main types of inversion have been described: type 1 (or distal) and type 2 (or proximal). The number of extragenic copies seems to be variable in the general population: inversions occurring in individuals with two distal or two proximal extragenic copies are called type 3 inversions.[2] Several other large structural changes of the FVIII gene involving intron 22 have already been reported, but the molecular bases have rarely been elucidated (Table 1). Intron 1 of the FVIII gene contains a 1041 bp region, which is also present telomeric to the gene in an opposite direction. An intrachromosomal recombination between the intronic and the extragenic copies results in intron 1 inversion.[3] Both intron 1 and intron 22 inversions prevent the formation of full-length FVIII mRNA and cause severe HA (FVIII activity<1%).

We introduced a combination of direct mutation detection and indirect marker analyses to provide carrier and prenatal diagnoses to Hungarian HA families.[4] In severe cases, screening for intron 22 inversions by Southern blotting was performed as the first line of investigation. In inversion-negative severe and in moderate to mild HA families, a set of three indirect markers (*Bcl*I, IVS13$(CA)_n$ and p39$(CA)_n$) was used for molecular diagnoses. Of the 138 participating families, 104 were affected by severe HA, from which we identified 4 rare variants of intron 22 rearrangement by Southern blotting. The goal of the present study was to characterize the large structural changes of the FVIII gene

Table 1. Reported intron 22 rearrangements of the FVIII gene detected by Southern blotting of *Bcl*I-digested genomic DNA samples, not corresponding to type 1 to 3 inversions.

BclI restriction profile	*Publication*
16, 14	Schröder *et al.*,[10] Weinmann *et al.*,[16] this report
16, 15.5	Windsor *et al.*[9]
16, 15.5, 14	Enayat *et al.*,[17] Schröder *et al.*[10]
16.5, 16,14	This report
17.5, 14	Poon *et al.*[14]
17.5, 16, 14*, 12°	Naylor *et al.*[15]
17.5, 16, 14	Yamazaki *et al.*[18]
17.5, 16, 15.5, 14	Schröder *et al.*[10]
19,17,16,14	Weinmann *et al.*[16]
20	Schröder *et al.*[10]
20, 17.5, 14 + an extra short band	Ljung *et al.*[19]
20, 17, 16, 14	This report
21.5, 16, 15.5, 14	Windsor *et al.*[9]
21.5, 16, 6.5	Strmecki *et al.*[20]
21.5, 20, 16, 14	Arruda *et al.*,[7] Windsor *et al.*,[9] this report
21.5, 20, 17.5, 14	Enayat *et al.*[21]
22, 16, 15, 14	Ljung *et al.*[19]
22, 21, 16, 14	Strmecki *et al.*[20]

**The band showed an approximately doubled intensity compared to other bands.*
°The band was detected by a different probe from the homologous region, but did not hybridize with the 0.9 kb EcoRI/SacI fragment from plasmid p482.6.

observed in these variant cases to provide the molecular bases for precise genetic counselling and to test for the presence of intron 1 inversion among intron 22 inversion negative cases.

Design and Methods

One hundred and ninety-one subjects from 104 families affected with severe HA were investigated for the presence of FVIII gene inversion. Patients and their family members were referred to the National Hemophilia Center for genetic counselling. The phenotypic diagnosis of HA and the classification (i.e. severe, moderate or mild) was established by standard coagulation assays (essentially by the determination of plasma FVIII activity).

Genomic DNA was isolated from anticoagulated peripheral blood samples by the standard salting out procedure.[5] Southern blots of *Bcl*I digested genomic DNA were carried out by standard procedures using the 0.9 kb EcoRI/SacI fragment from plasmid p482.6 (ATCC cat. no. 57203) as a hybridization probe.

Intragenic and extragenic intron 22 homologous regions were amplified simultaneously with four primers (P, Q, A, B) and cycling conditions in a multiplex long-distance PCR (LD-PCR), as reported by Liu *et al.*[6] In variant cases, the origin of PCR products was examined by separate amplifications with primer pairs P-Q, P-B, A-Q and A-B. Intron 1 inversion was investigated with primers described by Bagnall *et al.*[3] FVIII exon 22- and 23-specific PCR amplifications of genomic DNA were performed using primers and conditions described by Arruda *et al.*[7] Exons 14, 16 and 26 were amplified according to Diamond *et al.*[8] The deletion of either exon was established as the loss of specific PCR product on a 2% agarose gel together with appropriate positive and negative controls.

Results

Upon screening 104 unrelated families, we found intron 22 inversion in 54 (52%) cases. Forty-three cases (80%) showed distal inversion and 11 cases (20%) showed proximal type inversion. Unusual Southern blot patterns occurred in four of the families studied. Carrier and prenatal diagnoses were requested in 52 families, of which 32 families were affected by intron 22 rearrangements. From the 32 families, 10 at risk women were diagnosed as carriers, and 22 were diagnosed as non-carriers. Beside the 10 diagnosed carriers, the direct identification of the inversion mutation allows an accurate and rapid prenatal diagnosis in the future for 16 obligate and 12 sporadic carriers. Prenatal determination of the presence of intron 22 inversion was performed in five male fetuses. During screening for intron 1 inversion among 43 patients, who were intron 22 inversion negative, we identified only wild type individuals.

Further investigations were undertaken in order to characterize the underlying mechanism in the four patients with unusual Southern blot patterns. The results of the *Bcl*I-Southern blots, the exon 22 and 23 amplifications, the multiplex LD-PCR (with primers P, Q, A and B) and the separate LD-PCR (with primers P and Q) are shown in Figure 1, panels A to D.

Case #1. The patient is a 5-year-old boy with severe HA. Inhibitor development has not been observed during replacement therapy. The maternal grandfather was also affected by HA. On the Southern blot, the 21.5 kb *Bcl*I fragment was absent and the 16 and 14 kb bands with equal intensity remained unaltered (Figure 1A, lane V1). Specific amplifications of FVIII exons 26, 23, 22, 16 and 14 showed a deletion, affecting exons 16 and 22, while exons 14, 23, and 26 seemed to be intact (exon 22 and 23 amplifications are shown in Figure 1B, lane V1). By multiplex LD-PCR, only primers A and B gave

a 10 kb PCR product, and the 12 kb long P-Q band corresponding to the intronic homologous region was absent (Figure 1C, lane V1).

Case #2. The patient (aged 18) with severe HA had high-titer inhibitor against the FVIII protein. The family history indicated that the mother and the maternal grandmother were obligatory carriers. Southern blot analysis of *Bcl*I-digested DNA of the patient showed three bands (16.5, 16 and 14 kb) of equal intensity. A combined pattern (of 21.5, 16.5, 16 and 14 kb) was seen in the patient's mother (Figure 1A, lane CV2). In multiplex LD-PCR only primers A and B gave a 10 kb PCR product (*not shown*), an identical pattern as seen for V1. The lack of the specific PCR product for FVIII exons 23 and 26 (Figure 1B, lanes V2) proved the existence of a large deletion, which starts from intron 22 and involves the last four exons of the FVIII gene.

Case #3. This patient with severe HA (aged 48) with a single affected brother in the family had no history of inhibitor development. Southern blot analysis of *Bcl*I-digested DNA indicated four bands (20, 17, 16 and 14 kb, see Figure 1A, lane V3) of equal intensity for the affected brothers. The size of the extra band (approximately 17 kb) was clearly smaller than the 17.5 kb characteristic of distal inversion as indicated in Figure 1A (lane V3). LD-PCR showed an unusual pattern of three bands: unaltered intronic and extragenic copies with an inversion-affected copy of the homologous region (12, 11 and 10 kb, Figure 1C, lane V3). Separate amplifications with different primer combinations were set up to determine the origin of the different PCR fragments. Primers P and Q gave the 12 kb band (Figure 1D, lane V3), while amplification with primers A and B resulted in the 10 kb band. An 11 kb band was amplified with primers P and B, whereas primers A and Q gave no PCR product (*not shown*).

Case #4. The patient (aged 17) with severe HA had an affected maternal grandfather. Four bands of equal intensity were detected by Southern blot analysis: an intact and an inversion-affected intronic band, together with two unaltered extragenic bands (21.5, 20, 16 and 14 kb, Figure 1A, lane V4). Multiplex and separate LD-PCR amplifications (Figure 1C and 1D, lanes V4) gave the same results as those observed in case #3 (12 kb band with primers P and Q, 10 kb band with primers A and B, and an 11 kb band with primers P and B, but not with primers A and Q). Amplifications of exons 14, 16, 22, 23 and 26 showed intact coding regions in cases #3 and 4 (Figure 1B, lanes V3 and V4). None of the four variant cases carried the intron 1 inversion.

Discussion

Screening for intron 22 inversion has become a routine, first-line investigation for carrier detection and prenatal diagnosis in the care of severe HA. The reported frequency of intron 22 inversion in different countries varies from 28% to 53%. We found that 52% (54/104) of the Hungarian severe HA patients in the current study had type 1 or type 2 inversion. The combined frequency and relative proportion of these inversions (80% and 20% respectively) are consistent with those reported in other studies.

*Bcl*I-restriction profiles different from normal and from type 1 or type 2 inversions were reported to have a frequency as low as 1.2% (25/2093) in a large international study.[2] Forty percent of these rare patterns belonged to type 3 inversions, while the remaining cases have not been characterized in detail. In our series, four patients showed Southern blot patterns differing from type 1 to 3 inversions. This is an incidence of 3.8%, higher than that reported by Antonarakis *et al.*,[2] but similar to that found by Windsor *et al.*[9] and by Schröder *et al.*[10] Several variant patterns, not corresponding to type 1 to 3 inversions, have already been described (Table 1).

In two of our four variant cases, severe HA was caused by large deletions involving several coding exons and intron 22 to different degrees. More than 100 different large deletions (of 2 to 210 kb) of the FVIII gene have been reported so far in the database.[11] In case 1, the deletion affected exons 16-22 and intron 22 (probably including the hybridization site of the probe). The same *Bcl*I restriction profile was reported by other authors (Table 1). It is interesting that their cases showed deletions extending from intron 22 at least to exon 16, as in our patient.

In case 2, the observed *Bcl*I restriction pattern (16.5 kb, 16 kb and 14 kb bands) initially suggested the presence of an extra *Bcl*I restriction site in the normal 21.5 kb fragment. In the literature, only a single intron 22 *Bcl*I restriction site variant has been reported in non-hemophilic individuals of an affected family.[12] Exon amplifications and LD-PCR proved the existence of a large deletion, starting from intron 22 and extending at least to exon 26 in this case. Such a Southern blot pattern has not been reported in the literature previously. Although patients with deletions of exons 23-26 are listed in the database, they were investigated before the intron 22 inversion mutation was described.[13]

The remaining two patients with unusual patterns showed four homologous regions in the genome. In case 3, the loss of the 21.5 kb intronic band and the presence of the typical 20 kb inversion-affected intronic band indicated that a variant type of inversion caused the disruption of the FVIII gene. The *Bcl*I restriction profile resembled type 3 inversion, although the aberrant extra band was smaller (about 17 kb) than the typical inversion-affected distal fragment (17.5 kb). This type of inversion has not been previously described. Although the inversion mechanism does not usually result in either loss

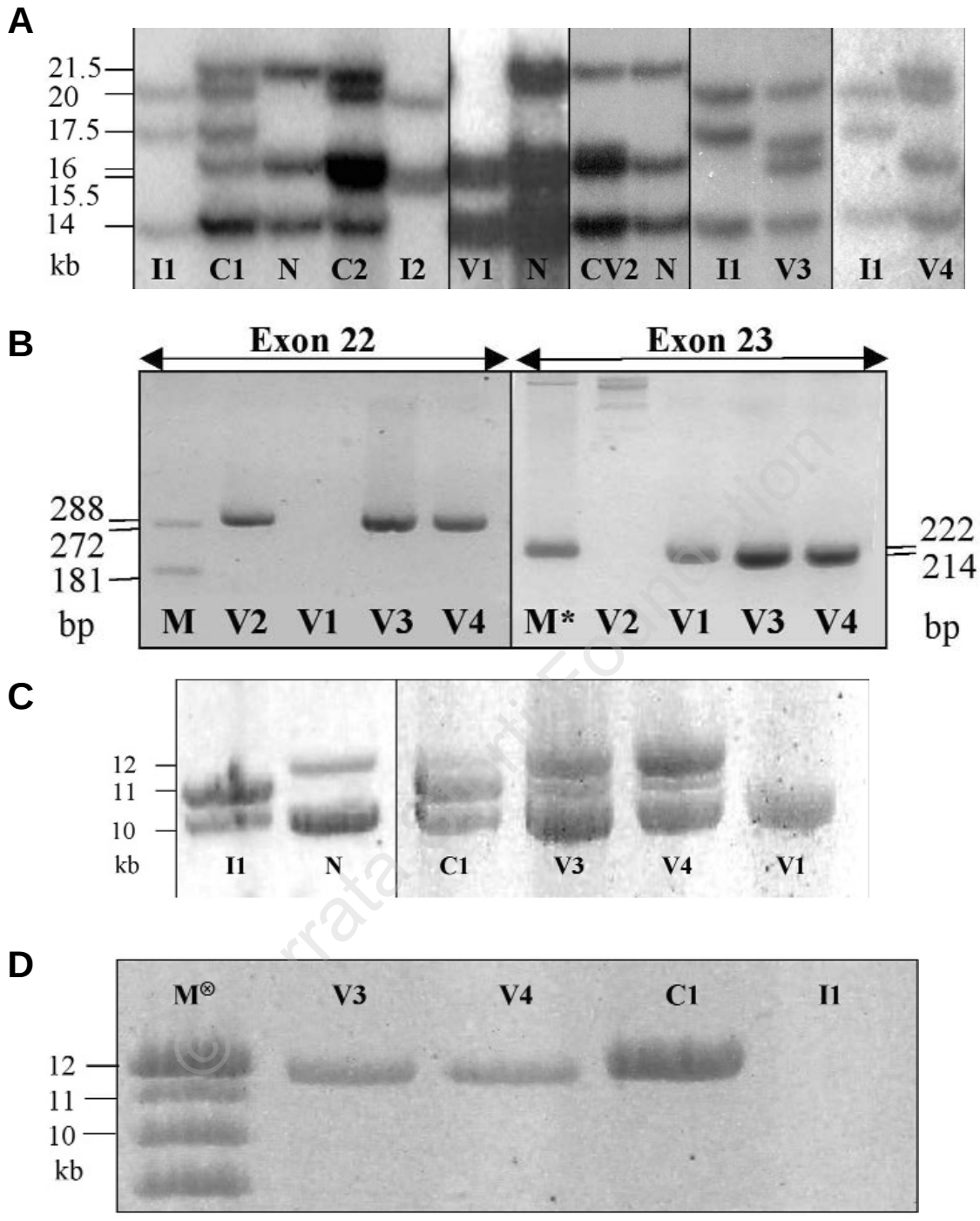

Figure 1. Results of Southern blotting, exon 22 and 23 amplifications, multiplex and separate LD-PCR in healthy individuals, in HA patients and carriers with intron 22 inversion and in the variant cases presented in the study. A: common and rare restriction profiles of *BclI*-digested genomic DNA samples. Southern blotting was performed by using a radiolabeled probe containing a fragment of the homologous region and autoradiography is shown; B: PCR amplification of exons 22 and 23. The PCR products were electrophoresed on 3% agarose gels and visualized by ethidium bromide staining. The expected sizes of the PCR products are 288 bp for exon 22 and 214 bp for exon 23; C: PCR product patterns of multiplex LD-PCR. The PCR products were electrophoresed on 0.6% agarose gels; D: Separate LD-PCR amplification of the intragenic homologous region with primers P I1 and QI2. The PCR products were electrophoresed on 0.6% agarose gels. N: normal pattern; type 1 (distal) inversion; male HA patient; C1: female carrier. Type 2 (proximal) inversion; male HA patient; C2: female carrier; V1, V2, V3, V4: variants detected in cases 1, 2, 3 and 4; CV2: variant female carrier (mother of case 2). Fragment sizes are indicated on either sides of the panels in kilobases (kb) or base pairs (bp). The following size markers were used: M: 272 and 181 bp, M*: 222 bp (panel B); M: 12, 11, 10 kb (panel D).

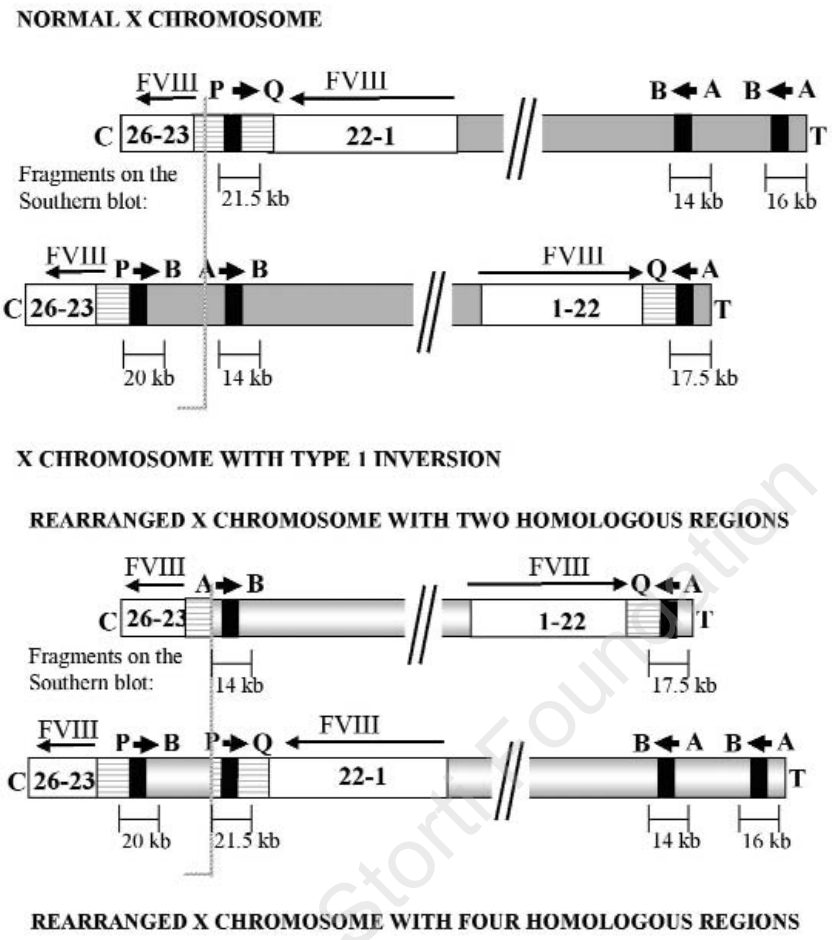

Figure 2. Hypothetical etiopathogenetic mechanism for the intron 22 rearrangement, observed in the presented cases with 4 homologous copies (cases #3 and #4). The figure is not drawn to scale. Complementary sites of the primers used in the long distance-PCR are marked with the appropriate letters (P, Q, A or B) and the expected fragment lengths (in kilobases) on Southern blot are shown under each chromosome. A, Misalignment of a normal and a distal inversion-affected X chromosome. The intronic homologous region of the normal X chromosome and the non-affected extragenic homologous region of the distal inversion-affected X chromosome are involved. B, Recombination outside of the homologous region resulting in two different rearranged X chromosomes with two or four homologous regions. The breakpoint is marked with the dotted line. The Southern blot pattern of 17.5 and 14 kb found on the rearranged X chromosome with two homologous regions was previously reported by Poon *et al.* [14] The Southern blot and LD-PCR patterns found on the rearranged X chromosome with four homologous regions was observed in one of the variant cases of the current study (case #4). We hypothesize that a similar recombination occurred in our other variant case with four homologous regions (case #3), but the position of the breakpoint was closer to the P-Q region affecting the 21.5 kb Southern blot fragment. FVIII: factor VIII gene; white boxes marked with *1-22* and *23-26*: different exons of the factor VIII gene; black boxes: homologous regions; striped boxes: intron 22 outside of the homologous region; P, Q, A, B: primers used in long-distance PCR (LD-PCR); regular arrows: direction of transcription of the factor VIII gene; bold arrows: direction of transcription of the FVIIIA gene located in the different homologous regions; dotted line: possible breakpoint outside of the homologous region; C: centromere; T: telomere; kb: kilobases.

or gain of DNA, we hypothesized that a type 3 inversion and a concomitant deletion at the site of inversion junction occurred. Truncation of either homologous region involved in inversion may prevent LD-PCR amplification with primers P-B or A-Q. Taking everything into consideration, in case 3, we expected to find the inversion-affected band (11 kb), the non-recombined extragenic band (10 kb) and the possible loss of amplification either with primers P-B or with primers A-Q in LD-PCR. Beside the expected bands, primers P and Q amplified a 12 kb band, suggesting that both an intact and an inversion-affected intronic homologous copy is present in the genome. The presence of an intact intronic band (a 12 kb band with the P-Q primer pair by LD-PCR) in the genome of case 3 rules out the possibility that

this variant represents the common type 3 inversion. The same LD-PCR results in cases 3 and 4 suggest similar molecular backgrounds for the two different Southern blot patterns.

The results in both cases are unusual because (i) the number of homologous extragenic repeats is reported to be polymorphic, but the number of intronic copies is not; (ii) one, rather than two, homologous region seems to be affected by inversion. The coexistence of an intact and an inversion-affected intronic homologous region on a single X chromosome could be explained by a recombination event between a distal inversion-affected and a normal X chromosome (Figure 2A). If a recombination occurs between the intact extragenic copy of a distal inversion-affected chromosome and the intronic copy of a normal X chromosome, and the breakpoint lies outside the homologous region (Figure 2B), one of the chromosomes will contain 4 homologous regions corresponding to PB, PQ, and 2 AB fragments in the LD-PCR, and to 21.5, 20, 16 and 14 kb fragments on the Southern blot (as in case 4). The other chromosome will contain AB and AQ fragments in the LD-PCR and 17.5 and 14 kb fragments on the Southern blot. The occurrence of this second variant was described by Poon *et al.*[14] We hypothesize that a similar recombination occurred in case 3, but that the breakpoint lay closer to the PQ region affecting the 21.5 kb Southern blot fragment.

Although our proposed mechanism can explain both of our variant cases with four homologous regions, further investigations are required to rule out other possibilities. Naylor *et al.*[15] suggested that alternative mechanisms, such as transposition, could also generate extra copies of the homologous region. They described a patient with an extra truncated copy located about 200 kb telomeric from FVIII. Alternative hybridization probes from other parts of the homologous region may confirm or exclude the presence of additional truncated homologous copies in the genome, which may remain undetected by Southern blot and LD-PCR; if due to a potential truncation the recognition sites of the probe and/or the primers are deleted. According to our proposed mechanism the sequence and the direction of the exons are not affected, only intron 22 is disrupted by the insertion. An alternative inversion assay, using mRNA to detect the failure of the reverse transcription-PCR assay crossing the exon 22 and 23 boundary, would be necessary to detect the lack of the full-length FVIII mRNA. Unfortunately, the mothers and the grandmothers of cases 3 and 4 were not available for study. The proposed misalignment between a normal and a distal inversion affected X chromosome (Figure 2) may occur during female gametogenesis in a carrier with distal inversion. The presence of the common distal but not variant inversion in female relatives of older generations of cases 3 and 4 would prove our hypothesis.

Intron 1 inversion was reported to occur at a frequency of 4.8% among severe HA patients, and 8.7% among severe HA patients not affected by intron 22 inversion. FVIII gene haplotype analyses demonstrated that intron 1 inversions have occurred independently and that a founder effect could be ruled out.[3] Among our intron 22 inversion negative severe HA patients (n=43) we identified only wild type individuals. This shows that intron 1 inversion is not common in Hungary, but the difference, possibly due to the small number of investigated individuals, is not significant (p=0.06).

The unusual cases of the present study further support the suggestion that FVIII intron 22 represents a hot spot for large gene rearrangements. Illegitimate cross-over events characteristic of this distal region of X chromosome, generated by the homologous regions, are not only reasons for the increased incidence of inversions, but also for the presence of deletions and duplications involving intron 22 of the factor VIII gene.

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Contributions

All authors of this manuscript contributed to the conception and design, analysis and interpretation of data; drafting of the article and revision of it; all gave final approval of the version to be published. The authors thank Horváth Csongorné and Pfundt Antalné for technical assistance. The help of Balázs Sarkadi in the critical review of the manuscript is also gratefully acknowledged.

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Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Paul Giangrande, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Professor Giangrande and the Editors. Manuscript received December 11, 2002; accepted May 28, 2003.

In the following paragraphs, Professor Giangrande summarizes the peer-review process and its outcomes.

What is already known on this topic

The intron 22 inversion is the common set cause of severe hemophilia, accounting for approximately half of all cases. It is also associated with an increased risk of inhibitor development. Inversions in intron 1 have also been recently reported as a relatively frequent underlying cause, although data on the incidence and associated risk of inhibitor development are less clear at present.

What this study adds

This study reports new variants of the intron inversions and some evidence that the site is a hotspot for new mutations. Information on the incidence of intron 1 inversions is reported, although the number studied is small and it is difficult to extrapolate these data.