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New Insight into the Molecular Basis of Hemophilia A

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Abstract

Since publication of the sequence of the factor VIII gene (*F8*) in 1984, a large number of mutations that cause hemophilia A (HA) have been identified. With the technical advances associated with mutation screenings, it is now possible to identify a putative *F8* sequence alteration in the great majority of HA patients. The mutation spectrum includes 2 inversion hot spots (intron 1 and intron 22 inversions) mediated by intrachromosomal recombination between 2 copies of long inverted repeats, one of which lies within the *F8* gene whereas the other is extragenic. Point mutations are distributed over all of the exons, and deletions or insertions of different sizes and mutations affecting splice sites account for the rest of the known mutations. In a small number of cases, however, we are unable to find any disease-determining DNA changes in the coding regions of the *F8* gene. This fact points to possibilities of unknown gene rearrangements that disrupt the *F8* gene or mutations in other genes that play a role in the processing/secretion of the factor VIII protein. Moreover, the proof of an absence of *F8* messenger RNA (mRNA) in one patient points to either a defect in the expression of *F8* mRNA or its rapid degradation, which may represent a novel mechanism leading to HA.
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Key words: Factor VIII; Hemophilia A; Mutations; mRNA; Expression

1. Introduction

Classic hemophilia A (HA) is caused by an absence or deficiency of factor VIII (FVIII) protein activity (FVIII:C). Clinically, the disease is classified as severe (<1% FVIII:C), moderate (1% < FVIII:C < 5%), or mild (>5% FVIII:C), depending on the residual FVIII activity. Bleeding occurs mainly in the joints and muscles but can also affect internal organs. HA is a lifetime disease that is transmitted from usually asymptomatic carrier females to their sons. The FVIII protein, which circulates in the plasma in trace amounts (100-200 ng/mL) [1], plays a central regulatory role in the hemostatic network: it accelerates the proteolytic activation of FX by activated FIX on platelet surfaces. During this process, FVIII provides a structural support for the interaction of FX with activated FIX. The molecular causes of FVIII deficiency can be divided into 3 main categories. The first category includes classic mutations in the *F8* gene that cause structural changes in the FVIII molecule or even produce a truncated protein lacking essential functional domains. The second category comprises mutations in proteins that interact intracellularly in the correct folding and trafficking of the FVIII protein [2-5] or mutations in extracellular plasma proteins such as von Willebrand factor (VWF) [6-9]. The third category addresses patients who have clinical HA but have no detectable mutations in the *F8* gene or in any of the known interacting partners. Evidence from at least one patient sample indicates that low or no expression of *F8* messenger RNA (mRNA) or its rapid degradation may be the cause of the HA.

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1.1. The F8 Gene

The *F8* gene was cloned in 1984 by Gitschier and colleagues at Genentech [10]. The gene spans 186 kb of genomic DNA and codes for an mRNA of 9.1 kb. It is located at the most distal part of the long arm of the X chromosome (Xq28). The *F8* gene consists of 26 exons, 24 of which vary in size from 69 to 262 bp (Figure 1). The remaining 2 exons (exons 14 and 26) are relatively large at 3106 bp and 1959 bp, respectively. The introns vary widely in size between 200 bp and 32.5 kb. Of particular interest is the large intron 22, which contains a 9.5-kb region 5.78 kb downstream from exon 22, which has been designated by

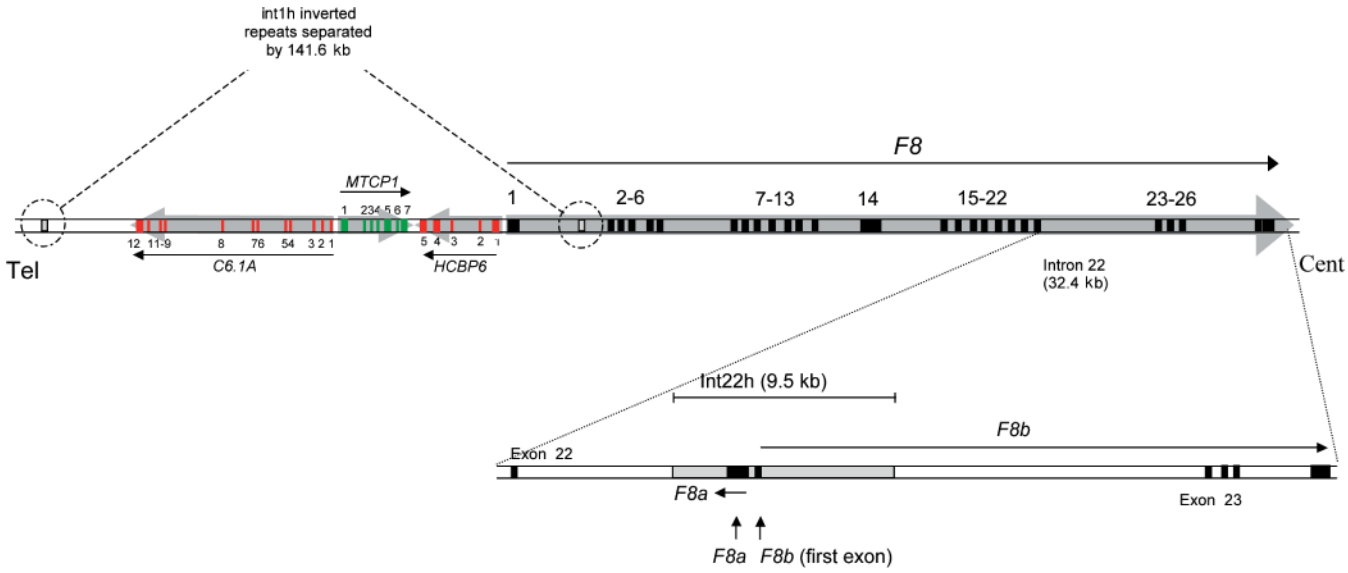

Figure 1. *F8* genomic organization at Xq28. A few neighboring 5′ genes are also shown. Transcription orientation is shown as horizontal gray arrows. The 2 intron 1 homologous region (int1h) repeats that are involved in intron 1 inversion are highlighted by open dashed circles. The *F8* intron 22 region is shown in a magnification to show the 2 transcripts that emerge from the int22h repeat. Tel indicates telomere; Cent, centromere.

Naylor et al [11] as the intron 22 homologous region (int22h-1). This region is present in 2 other copies approximately 497 kb distal to the 3′ end of the gene. Intrachromosomal homologous recombination between int22h-1 and either int22h-2 or int22h-3 during male meiosis results in the inversion of the intervening sequence, which interrupts *F8* mRNA at intron 22 (Figure 2). Most of these inversions have a paternal origin, because the presence of the second homologous X chromosome during oogenesis has a protective effect [12]. Another pair of inverted repeats, the int1h repeats, are present near exon 1 (int1h-1, 15.26 kb downstream of exon 1; int1h-2, 125 kb upstream of exon 1) (Figure 1). Again, an intrachromosomal recombination between the 2 repeats leads to an inversion of the intervening sequence, in this case resulting in a short *F8* mRNA including only exon 1.

A surprising observation is that 2 transcripts emerge from the int22h-1 region. The first is that of *F8a*, a small intronless gene that is transcribed in the opposite direction of the *F8* gene [13]. The second transcript is for *F8b*, which is transcribed in the same direction as the *F8* gene and shares the last 4 exons (exons 23-26) [14]. A CpG island that plays the role of a bidirectional promoter separates the 2 genes. Transcripts corresponding to both genes have been detected in a variety of tissues. *F8b* may not have an essential function, because HA patients with a gross deletion including the whole gene do not show any symptoms in addition to their hemophilia [15]. Also unknown is whether this transcript is translated into a protein that corresponds to the phospholipid-binding domain of FVIII. *F8a* codes for a 40-kd protein that interacts with huntingtin protein (thus, F8a is also called huntingtin-associated protein or HAP40) [16]; however, the biological significance of this interaction is not fully understood.

1.2. The FVIII Protein

The main expression sites for the FVIII protein are sinusoidal endothelial cells, Kupffer cells, and, to a lesser extent, hepatocytes in the liver. In addition, glomeruli and tubular epithelial cells in the kidney have also been found to show some expression. However, secretion of the FVIII protein has been demonstrated only in cells of hepatic origin [17]. The primary product is a single polypeptide chain of 2351 amino acid residues, from which the first 19 amino acids, corresponding to a hydrophobic signal peptide, are removed during secretion. Three main types of internal domains can be distinguished in the mature protein: 3 A domains, a large B domain, and 2 C domains (Figure 3) [18]. The B domain at 908 residues is the largest. The sizes of the A1, A2, and A3 domains are 336, 345, and 335 amino acid residues, respectively; the C1 and C2 domains comprise 155 residues and 152 residues, respectively. Three small acidic domains, designated a1, a2, and a3, connect, respectively, the A1 and A2 domains, the A2 domain to the amino-terminal portion of the B domain, and the carboxy-terminal portion of the B domain to the A3 domain. The resulting domain organization for the single-peptide FVIII protein is A1-a1-A2-a2-B-a3-A3-C1-C2 [18].

Before FVIII secretion, an as yet unknown factor (although PACE/furin proteins may have a role) cleaves the peptide chain within the B domain to give a heterodimer consisting of a heavy chain and a light chain [19,20]. The heavy chain of 90 to 220 kd consists of the A1-a1-A2-a2 sequence of domains with variable extensions of the B domain, whereas the 80-kd light chain consists of the a3-A3-C1-C2 sequence of domains. The light and heavy chains are associated with each other via divalent cation–dependent interaction. In plasma, FVIII heterodimers circulate in a

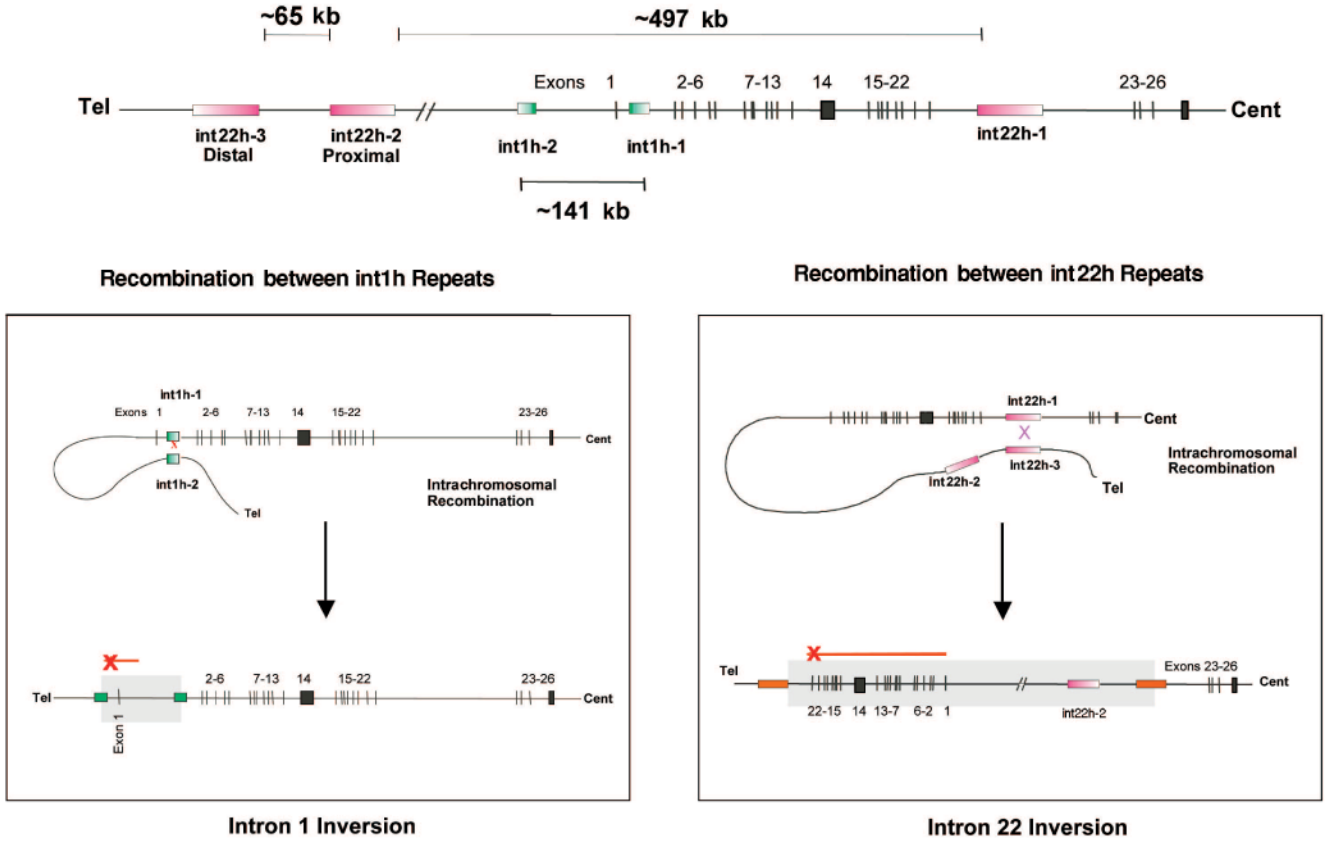

Figure 2. Mechanisms of intron 1 and intron 22 inversions. The 3 intron 22 homologous region (int22h) repeats (1, 2, and 3) and the 2 int1h repeats (1 and 2) are represented as shadowed rectangles. Their relative orientations to each other are represented by the scale of gray. A schematic representation of the process of homologous recombination and the inversion of the intervening sequence as an end product are also shown. Tel indicates telomere; Cent, centromere.

noncovalently bound complex (mainly via the FVIII a3 domain) with the giant VWF protein, which functions as a carrier in the blood for the inactive FVIII molecule [21]. Moreover, VWF protects FVIII from proteolytic activation and concentrates FVIII at sites of active hemostasis through binding to subendothelial matrix proteins and adherent platelets [22].

The FVIII heterodimer is activated by thrombin cleavage at 3 arginine residues: R372, R740, and R1689 [23]. Both cleavages at R372 and R1689 are essential for the activation of the protein, whereas the cleavage at R740, which removes the remaining part of the bulky B domain, is not essential. Therefore, the active FVIII protein is a heterotrimer comprising A1-a1, A2-a2, and A3-C1-C2. Deactivation of FVIII occurs via further cleavages by FX at residues R336 and R1721, by activated FIX at R336 and R1719, or by activated protein C at R336 and R562 [24,25] (Figure 3).

2. Mutations Causing or Contributing to the HA Phenotype

In a broad sense, the FVIII protein deficiency that leads to HA could be caused by a qualitative defect in the FVIII protein due to mutations in the FVIII protein itself (section 2.1, below). On the other hand, a quantitative defect that leads to little FVIII at injury sites would lead to the same clinical phenotype. The latter class of defects could be caused by mutations in transporter proteins involved in the secretion or correct folding of FVIII or by mutations in VWF, which is the FVIII carrier in the blood (section 2.2). Still remaining is a minority of patients with typical clinical HA who have no detectable mutations for either of these 2 classes of defects (section 2.3). In the following sections, we discuss these 3 categories in more detail.

2.1. Mutations in the F8 Gene

Classic HA is caused by mutations located in the *F8* gene. The spectrum of these mutations includes point mutations, various deletions and insertions, and the 2 common intron 1 and intron 22 inversions described above. The site of intron 22 inversion is a major mutational hot spot; such an inversion causes disruption of the *F8* gene and leads to severe hemophilia. This inversion accounts for up to 50% of severe cases and approximately 30% of all cases [12,26] (Table 1). To date, 897 unique mutations of different types (other than inversions) have been reported in the HA database (HAMSTeRS: http://europium.csc.mrc.ac.uk/WebPages/Main/main.htm). The 623 different point mutations are distributed over all of the exons. A second group of mutations comprises C-to-T

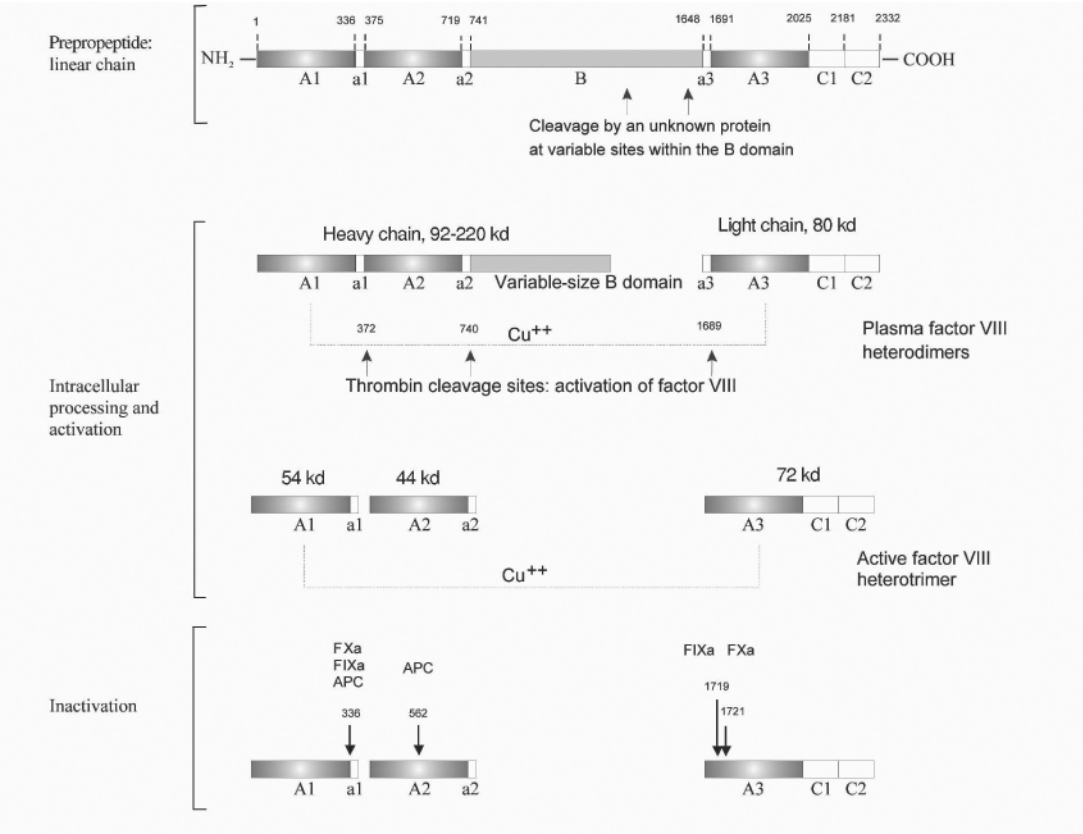

Figure 3. The factor VIII protein. The linear structure of the protein with the distinguishable domains is shown. Activation and deactivation sites are indicated by vertical arrows; the protein involved in the cleavage, if known, is also indicated. FXa indicates activated factor X; FIXa, activated factor IX; APC, activated protein C.

and G-to-A mutations at one of the 70 CpG dinucleotide hot spots; such mutations include those at codons 1689, 1941, 1966, 2147, 2150, 2159, and 2163. These mutations are caused by spontaneous deamination of methylated cytosines, which yields a uracil that will be recognized, if not corrected, as thymine during DNA replication [27,28]. There are 159 independent small deletions, which are distributed over all of the exons except exons 20 and 21. The 60 known small insertions are distributed over various exons; exons 3, 7, 10, 15, 16, and 23 currently have no reported insertion mutations. A third category of recurrent mutations is the insertion and deletion of As in A stretches that occur at different positions in the *F8* gene, with the main mutational hot spots being 9 As at codons 1191 to 1194, 8 As at codons 1439 to 1441, and 6 As at codons 1588 to 1590; all of these mutations are in exon 14. Moreover, up to 55 different gross deletions have been reported: the smallest such deletion covers one exon, and the largest includes the entire *F8* gene locus. The frequency distribution of mutations for 846 German HA patients is presented in Table 1 [26].

Table 1.
Summary of the Mutations in 846 German Hemophilia A Patients*

Mutation Type	Mutations, n (%)
Intron 22 inversion	302 (35.7)
Intron 1 inversion	8 (1)
Point mutations	402 (47.5)
Small deletions	64 (7.6)
Small insertions	23 (2.7)
Large deletions	25 (2.9)
Splice site mutations	22 (2.6)
Total	846 (100)

*See reference [26].

2.2. Mutations in Interacting Partners

After the single-chain prepeptide FVIII molecule is produced in the endoplasmic reticulum, it interacts with many proteins, some of which are intracellular and others are in the blood circulation. Mutations in some of these interacting partners have been reported to cause FVIII deficiency or a combined FV/FVIII deficiency that has an HA-like phenotype and can be mistaken for classic HA.

2.2.1. Intracellular Interaction

Although no detailed map of FVIII intracellular interactions with cellular proteins is available, some known interaction partners include immunoglobulin-binding protein (BiP), calnexin, calreticulin, lectin mannose-binding protein (LMAN1), and multiple coagulation factor deficiency 2 pro-

tein (MCFD2) [2-5]. The FVIII protein binds to the chaperones BiP, calnexin, and calreticulin in the endoplasmic reticulum. Binding to BiP is a strong interaction that requires the hydrolysis of adenosine triphosphate for release. On the other hand, calnexin and calreticulin bind the misfolded proteins and retain them in the endoplasmic reticulum. Because these 3 proteins exert a negative effect on the secretion process, a mutation in or an absence of these proteins may not necessarily cause an HA phenotype. In accordance with this fact, no known mutations in these proteins are associated with HA.

In the endoplasmic reticulum–Golgi intermediate compartment (known as ERGIC), the FVIII molecule interacts with the protein chaperone complex LMAN1/MCFD2, which facilitates FVIII transport from the endoplasmic reticulum to the Golgi compartment. Mutations in either of the chaperone complex subunits have been reported to cause combined FV/FVIII deficiency with a mild phenotype (reviewed in [29]). These findings indicate the importance of these chaperone proteins in the secretion pathways of FV and FVIII. Because both FV and FVIII levels are decreased in these cases, a misdiagnosis that would point to an FVIII mutation can be easily discarded.

2.2.2. Extracellular Interaction

Once secreted, FVIII is found associated with VWF, which functions as an FVIII carrier in the blood and protects it against the proteolytic action of activated protein C. The site of interaction between FVIII and VWF includes the a3 domain (amino acid residues 1649-1689) and 2 regions in the C2 domain (residues 2181-2243 and 2303-2332) on the FVIII molecule, and the amino-terminal domain (D′) and part of the D3 domain of the mature VWF protein (residues 1-272), corresponding to exons 18 to 20 of the VWF gene [30]. Mutations in either molecule that disrupt this interaction are expected to produce a hemophilia-like phenotype [6-9]. Indeed, mutations at the amino terminus of VWF, as in VWF type Normandy 2 (2N VWF), result in such a phenotype. However, unlike the classic hemophilia that is manifested in males, 2N von Willebrand disease is inherited in an autosomal recessive manner. To date, 17 different missense mutations that cause 2N VWF have been reported in the VWF mutation database (http://www.sheffield.ac.uk/vwf/). These mutations are distributed over exons 18 to 24, and the disease ranges in severity, depending on the mutation, from a severe phenotype (E787K) to moderate/mild phenotypes (T791M, Y795C, R816W) to a subhemophilic phenotype for the most common mutation (R854Q).

2.3. Unidentified Mutations

After all of the types of mutations described above have been excluded, the identification of a causative mutation that would explain the hemophilia phenotype is still not possible in a small percentage of patients (approximately 1.7% or 15/861 patients). We postulated in a recent study that some mutations could be located in the large introns of the *F8* gene and thereby cause abnormal splicing [31] or that some novel inversions or duplications of exons could disrupt the normal DNA sequence. To test these hypotheses, we performed a detailed analysis on *F8* mRNA isolated from whole blood. In 9 cases, we found normal splicing across all exon boundaries. A summary of the patient data and the results of this study are presented in Table 2.

Table 2.
Summary of Clinical Data for the Patients without Identified Mutations

Patient No.	Severity	Inhibitors	Mutation	Familial Case	*F8* Splicing
1	Severe	No	Not identified	Yes	Normal
43	Severe	No	Not identified	Yes	Normal
47	Severe	No	Not identified	Yes	Normal
51	Severe	No	Not identified	Yes	Normal
13	Severe	No	Not identified	No	Normal
16	Severe	No	Not identified	No	Normal
25	Severe	No	Not identified	No	Normal
29	Moderate/Mild	No	Not identified	No	Normal
44	Mild	No	Not identified	No	Normal

Moreover, we were unable to detect *F8* mRNA [32] in one of these patients. In a polymorphism analysis, we followed the expression pattern of the allele of interest in the patient and in the patient's mother and sister. The patient's allele was undetectable by reverse transcriptase–polymerase chain reaction analysis not only in the patient but also in both the mother and the sister. Figure 4 shows the pedigree

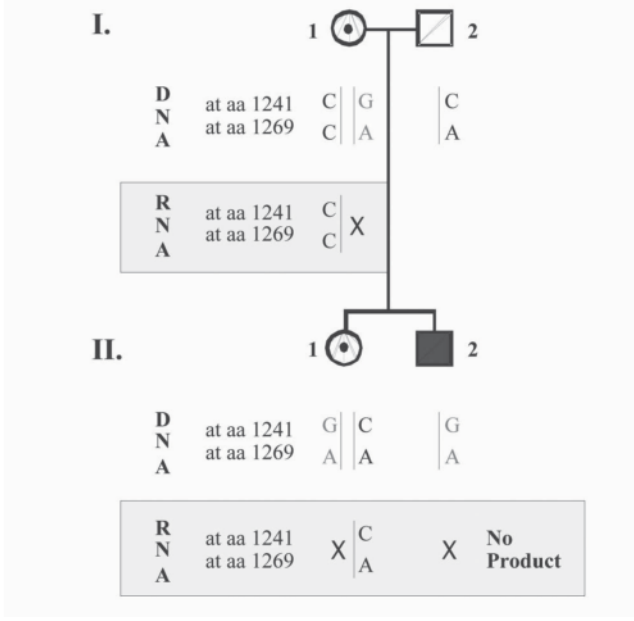

Figure 4. Pedigree of the hemophilia A family in which an *F8* allele segregates in a silent state in the mother, the sister, and the index patient. The filled square represents the index patient; open circles with a central dot represent obligate carriers (the mother and the sister). The *F8* alleles are distinguished on the basis of the common 2 polymorphisms at codons 1241 (C/G) and 1269 (C/A). The alleles in the studied individuals are indicated by vertical bars with 2 letters. The alleles detected from DNA and RNA analyses are listed below each individual. The GA allele that is not detected by reverse transcriptase–polymerase chain reaction analysis is represented by an *X*. aa indicates codon.

of the family and the segregation of the silent allele. At present, we do not know the mechanism behind the silencing of this allele in this family, and thus we cannot exclude the possibility that in the remaining unresolved cases (or at least in some of the cases) a quantitative expression problem contributes to the hemophilic phenotype. The reason or reasons for such low mRNA expression may be due to defects in some regulatory elements such as enhancers or other epigenetic factors; however, little is known about such factors for the *F8* gene.

One other possible explanation for this phenomenon (especially with respect to nonfamilial cases that do not necessarily show the typical X-linked inheritance) is that mutations in other proteins (other than the ones mentioned in section 2.2) may affect the proper folding, secretion, modification, and/or trafficking of the FVIII protein or modulate the activity of the mature protein in the blood. For example, the secretion efficiency of the FVIII molecule depends on the "correct" processing of the protein in the endoplasmic reticulum–Golgi apparatus. Such processing includes, but is not restricted to, phosphorylation, sulfatation, glycosylation, and proteolytic cleavage in the B domain at amino acid residues 1313 and 1648.

Most of the patients with no detectable mutations share 2 important characteristics: a severe clinical phenotype (7 of 9 patients) and no inhibitor development. The probability of inhibitor development has been shown to depend on the type of mutation, with a higher incidence of inhibitors associated with an absence of large parts of the FVIII protein due to nonsense mutations, gross deletions, or intron 1 and intron 22 inversions [33]. Thus, the absence of inhibitor formation in these patients is in line with the hypothesis that the defect in these patients is due to either inefficient secretion or inefficient expression of the FVIII protein.

3. Risk of Inhibitor Development and F8 Mutation Type

A major complication of FVIII replacement therapy for a hemophilic patient is the development of alloantibodies (FVIII inhibitors) against transfused FVIII. When this complication is a serious problem, as it is in approximately one third of patients with severe disease, FVIII substitution therapy is rendered ineffective. To date, many studies have investigated different parameters that increase the risk of inhibitor development, including genetic factors such as *F8* gene mutation, HLA genes, or the immune response genes. A major parameter that influences the risk of inhibitor development is the type of mutation in the *F8* gene (Table 3). Severe molecular gene defects, such as large deletions, intron 22 inversions, and nonsense mutations that cause a complete absence of the FVIII protein present a 7- to 10-fold higher risk for inhibitor formation than such genetic defects as small deletions, missense mutations, or splice site mutations that result in a loss of function but not a complete absence of the FVIII protein [34,35].

Recent studies of hemophilic siblings and hemophilic twins have mainly shown a high degree of concordance (78.3%, 195/249) between family members (ie, all affected members in a family are either inhibitor positive or inhibitor negative) [36]. The same study reported an even higher concordance (88.2%, 15/17) when only twins were considered. A more recent study has also analyzed the concordance in inhibitor formation among affected family members, and the results strongly suggest that immune response genes must have a great influence on the development of inhibitors [37]. In fact, the most recent study reveals a strong association between CA-repeat polymorphism in the promoter region of the interleukin 10 gene and the development of inhibitors [38].

Table 3.
Risk of Inhibitor Formation Depends on Extent of *F8* Gene Mutation

Mutation Type	Inhibitor Prevalence
Multidomain deletions	88%
All large deletions	41%
Light chain nonsense mutations	40%
All nonsense mutations	31%
Single domain deletions	25%
Intron 1 inversion	24%
Intron 22 inversions	21%
Non-A-run-small deletions/insertions	21%
Heavy chain nonsense mutations	17%
All small deletions/insertions	16%
C1-C2 missense mutations	10%
All Missense mutations	5%
Non C1-C2 missense mutations	3%
A-run-small deletions/insertions	3%

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