Absence or decreased activity of the F8 protein causes hemophilia A (HA) which is characterized by the occurrence of spontaneous bleeding. Mutations in the F8 gene, located at the telomeric end of the long arm of the X chromosome, have been identified as the cause of HA. Such mutations include the intron 22 and intron 1 inversions hot spots, point mutations (nonsense and missense mutations) that are distributed throughout all exons and various deletions and insertions [1]. For a review on mutation analysis and therapy refer to Graw et al. [2]. Recently, Oldenburg et al. [3] published the distribution of different types of mutations in the F8 gene (table 1). Patients with detectable mutations in the F8 gene account for a total of 98.2% of all patients. In the remaining 1.8% of pa-
Hemophilia A Patients With Undetectable Mutations

Previously, a selective group of 15 out of 860 patients was screened for all types of known mutations in the F8 gene, but no changes were detected [3]. However, in a recent data compilation based on 2,350 HA patients from Germany, we identified 53 probands (so far) in whom no mutation could be identified with any of the above mentioned techniques. With 9 of these patients we have been able to carry out more detailed research. Table 2 summarizes the present knowledge regarding these patients.

Most of the studied patients share 2 important characteristics: a severe clinical phenotype (7 out of 9) and no inhibitor development. The probability of inhibitor development is known to depend on the type of mutation with a higher incidence associated with the absence of large parts of the F8 protein due to nonsense mutations, gross deletions or intron 1 and intron 22 inversions [7]. Thus, the absence of inhibitor formation in these patients implies that some trace amount of F8 is present or that at least the endogenous F8 antigen was presented to the immune system at some stage during development. This suggest that the defects in these patients may be due to either insufficient secretion or expression of the F8 protein.

In 4 of the 9 families (all with severe HA), more than one male-hemophilic existed (table 2), thus favoring an X-linked inheritance. This assumption is strengthened by the fact that all 9 hemophiliacs were males. Five of the 9 families – 2 with non-severe and 3 with severe HA – have no history of hemophilia, making them candidates for a non-X-linked mode of inheritance. None of the family trees indicated consanguinity which could have triggered autosomal recessive conditions.

Taking the above observations into consideration, one could postulate a number of explanations for the hemophilia phenotypes in these patients we have been able to carry out more detailed analysis. The probability of inhibitor development is known to depend on the type of mutation with a higher incidence associated with the absence of large parts of the F8 protein due to nonsense mutations, gross deletions or intron 1 and intron 22 inversions [7]. Thus, the absence of inhibitor formation in these patients implies that some trace amount of F8 is present or that at least the endogenous F8 antigen was presented to the immune system at some stage during development. This suggest that the defects in these patients may be due to either insufficient secretion or expression of the F8 protein.

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To investigate the above hypotheses, we previously performed detailed mRNA analysis in patients available to us [8]. Detailed F8 analysis on the mRNA level would reveal the presence of rearrangements (such as inversions and duplications) that do not disrupt any of the exons themselves but rather change the relative exon positions or numbers. For example, a splicing defect that leaves out one or more exons in the mature mRNA due to an intronic mutation would be easily detected by sequencing the mRNA. A schematic diagram of the mRNA analysis is shown in figure 1.

Detailed mRNA Study

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Detailed analysis of cDNA by reverse transcriptase PCR (RT-PCR) represents a powerful tool for searching causative splicing mutations or gene rearrangements. This strategy is applied with disease-related genes in patients who do not show mutations in the coding regions at the DNA level [9, 10]. The same strategy was previously applied as the first-line mutation screening method for the F8 and F9 genes [11–13]. In fact, it was the detailed analysis of the F8 cDNA by Nayler et al. [14] that revealed a transcript interrupted in intron 22 in about 50% of severe hemophilia cases. This observation led to the discovery of a major gene rearrangement in the F8 gene – the intron 22 inversion [15, 16]. A similar experimental strategy led to the discovery of the intron 1 inversion that occurs at a worldwide frequency of about 4% of HA cases [17, 18].
In the group of patients with no detectable mutations, no abnormal splicing or rearrangements of the F8 cDNA were found (table 1). This indicates that the cause for HA in these patients may indeed lie in other yet unknown regulatory regions of the F8 gene or in genes encoding interacting/modifying proteins.

One possible explanation for this observation is that there may be mutations in chaperon proteins that affect proper folding, secretion, modification and/or trafficking of the F8 protein or modulate the activity of the mature molecule in the blood. Examples of such proteins are the ERGIC-53 (also known as LMAN1) and the recently identified MCFD2 protein, which are involved in transporting both F8 and F5 from the endoplasmic reticulum (ER) to the Golgi compartment and whose defects cause a combined F5/F8 deficiency [19, 20]. Since the F5 activity in these patients was normal (data not shown), involvement of these 2 particular proteins could be excluded. However, other as yet unknown proteins could be involved in F8 protein processing. For example, the secretion efficacy of the F8 molecule depends on correct processing of the protein in the ER-Golgi apparatus, which includes processes such as phosphorylation, sulfatation, glycosylation and proteolytic cleavage in the B domain at aa 1313 and 1648 by a yet unknown protein. Given that the B domain of the F8 is a target for 19 N-glycosylation sites and 3 O-glycosylation sites and taking into consideration the proposed role of this domain in the intracellular trafficking of F8 molecules, the involvement of glycosylation defects in the hemophilic phenotype is not unlikely.

Another possible explanation for the hemophilic phenotype is a diminished expression/secretion of the F8 mRNA/mature protein, which would lead to lower amounts of active F8 protein in circulation. In fact, 1 patient showed direct evidence of the absence of detectable F8 mRNA [21]. The reduced mRNA expression could be explained by defects in regulatory elements, such as enhancers or other epigenetic factors. However, with F8, little is known about such factors. Some unexplained silencing of expression has also been observed in other diseases, such as von Willebrand disease (VWD; www.sheffield.ac.uk/vwf/). VWD is a recessive disease that is usually caused by 2 mutations on both alleles of the von Willebrand factor (VWF) gene. However, a number of cases have been reported in which one allele harbored a mutation while the other allele is not expressed [22–24]. Congenital hypothyroidism is another recessive disease caused by mutations in the gene coding thyroid peroxidase (TPO). Patients suffering from this disease are usually homozygous or compound heterozygous for gene mutations. In about 17% of cases, there is only one mutated allele. In one of these cases, Fugazzola et al. [25] showed that on the DNA level the intact allele is unexpressed and on the RNA level it is undetectable. In all above cases, the exact molecular mechanisms leading to absent or decreased expression are yet unknown.

**Future Direction**

For further identification of the mutations in these HA cases, the following directions could be followed. Firstly, a quantitative RT-PCR approach is required to investigate whether these patients exhibit lower expression levels compared to healthy individuals. Secondly, an international consortium study is needed to assemble all familial HA cases with unidentified mutations and conduct a genome-wide linkage analysis to identify the defective locus (or loci). The phenotypic data of these patients will be completed with respect to the inhibitor status, potentially revealing a complete lack of endogenous F8 synthesis and thus indicating a severe molecular defect. Pedigree data will be gathered to identify patients with either sporadic or familial HA. Data on related female carriers will be included to increase the number of informative individuals. In families with familial HA (more than one hemophiliac or one hemophiliac plus at least one supposed carrier), F8 gene polymorphism will be analyzed to investigate whether the defect is segregating with the F8 gene. In families in which the phenotype does not segregate with the F8 gene, a complete genome linkage analysis will be carried out to identify chromosomal regions of interest that may harbor candidate genes that could potentially play a role in the pathogenesis of HA. Regions with significantly increased lod scores will be mapped in more detail to narrow down the candidate intervals. Genes within these intervals will be arranged according to function and size. Promising candidates will be sequenced to screen for mutations.
Conclusions

In a minority of HA patients, the molecular defect is not located in any of the genes known to be involved in the F8 pathway. Detailed RNA analysis in a group of these patients revealed normal F8 mRNA. Thus, we can exclude major rearrangements, such as novel inversions or duplications, that could disrupt the mRNA. Major efforts are still needed to identify the loci responsible for the HA phenotype in these patients.

References