

# Genetic Linkage of Two Intragenic Restriction Fragment Length Polymorphisms with von Willebrand's Disease Type IIA

## Evidence for a Defect in the von Willebrand Factor Gene

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### Abstract

Restriction fragment length polymorphisms (RFLPs), using the enzymes Bgl II and Xba I in conjunction with human von Willebrand factor (vWF) cDNA probes, have been described previously. In the present study we demonstrate the localization of both genetic markers within the vWF gene. The RFLPs were used to study the segregation of alleles associated with von Willebrand's disease (vWD) type IIA in a comprehensive, affected family. Individuals of this family were tested for their bleeding time and their plasma was analyzed for vWF antigen concentration and vWF ristocetin-cofactor activity. Based on these data, the affected members were diagnosed as vWD type-IIA patients; this conclusion was confirmed by the analysis of the multimeric vWF pattern of some of the patients. It was demonstrated that both RFLPs are completely linked with the vWD type-IIA trait. From this finding, we conclude that the defect that causes the vWD type IIA is most likely due to a mutation in the vWF gene and not to a mutation in a gene involved in posttranslational processing of the vWF protein.

### Introduction

The von Willebrand factor (vWF)<sup>1</sup> is a glycoprotein which circulates in plasma as a series of multimers with a relative mass of 0.5–20 × 10<sup>6</sup> (1, 2). vWF participates in the adhesion of blood platelets to the subendothelium after a vascular injury (3), and is essential for normal platelet plug formation, a process which is mediated more effectively by large size multimers than by small size oligomers. The vWF also serves as a carrier protein for the coagulation protein Factor VIII (FVIII), thereby forming the FVIII-vWF complex. Also, vWF apparently modulates the FVIII-vWF turnover (4).

A defect in vWF, or an abnormal plasma concentration of

this protein, results in a bleeding disorder known as von Willebrand's disease (vWD). This hereditary disorder may be due to a mutation in the vWF gene or in a gene involved in post-translational processing of vWF. The disorder is characterized by a prolonged bleeding time, decreased or normal vWF antigen (Ag) concentration, reduced ristocetin-cofactor activity (vWFRco activity), and a decreased or normal FVIII concentration and FVIII-procoagulant activity. Different types of vWD have been described which are classified by their antigen level, vWFRco activity, the pattern of organization of the plasma and platelet vWF multimers, and by the response of platelet-rich plasma to ristocetin (5). For example, patients with autosomal, dominantly inherited vWD type IIA display a decreased vWFRco activity and lack large and intermediate size multimers (2). As yet, the cause for this abnormal vWF protein is not known. In this study, we want to determine whether the vWD type IIA is caused by a mutation in the vWF gene or by a cellular defect in the vWF processing. The latter option would imply a decreased or abnormal synthesis or release of vWF from endothelial cells.

Recently, we and others have constructed plasmids containing partial- (6–10) and full-length vWF cDNA (11, 12) and have used vWF-cDNA probes with a panel of somatic cell hybrids to localize the gene on chromosome 12 (7, 8). The complete vWF cDNA includes an "open" reading frame of 8,439 nucleotides, encoding a precursor protein with a calculated molecular weight of 309,000 (unglycosylated). The precursor protein is proteolytically processed to yield two distinct proteins, i.e., vW AgII and the mature vWF subunit (11–13). The availability of vWF-cDNA probes has led to the identification of three restriction fragment length polymorphisms (RFLPs) (14–16). In general, RFLPs can be used as a tool in prenatal diagnosis and carrier detection, as shown for a number of genetic diseases (17–20). The polymorphisms located within the vWF gene, but which are most probably unrelated to the genetic defect, may allow gene tracking in families affected with vWD. Moreover, such genetic markers can be useful tools for the prenatal diagnosis of severe forms of vWD.

In this paper, we have established a restriction map of a segment of the vWF gene containing two of the three polymorphic sites. As a first application of two of these RFLPs, we have studied the genetic linkage of both RFLPs with the vWF allele, which is associated with vWD type IIA, in a three-generation family affected with vWD type IIA. It is concluded that the genetic defect, which results in vWD type IIA, is linked with both intragenic RFLPs.

### Methods

*vWD type-IIA family.* We investigated 39 individuals of a three-generation family affected with vWD. The affected members of this family

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1. *Abbreviations used in this paper:* GPIB, glycoprotein IB; RFLP, restriction fragment length polymorphism; 1× SSC, 0.15 M sodium chloride, 15 mM sodium citrate; vWF Ag, von Willebrand factor antigen; vWD, von Willebrand's disease; vWFRco activity, ristocetin-cofactor activity; FVIII, Factor VIII.

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have been diagnosed as vWD type IIA. Blood was collected in sodium citrate (final concentration 10.9 mM). For different determinations related to the vWF protein, the blood was centrifuged for 15 min at 3,000 g at 4°C and the plasma was separated. vWF Ag was assayed according to the electroimmunodiffusion method of Laurell (21) and the vWFRco activity was measured using formalin-fixed platelets as described (22). Bleeding times were determined using the technique of Ivy et al. (23). The multimeric organization of the vWF was analyzed by electrophoresis on a 1.4% agarose gel in the presence of 0.1% SDS (24). The vWF multimers were identified after incubation of the dried agarose gels with <sup>125</sup>I-rabbit anti-human vWF IgG and subsequently visualized by autoradiography.

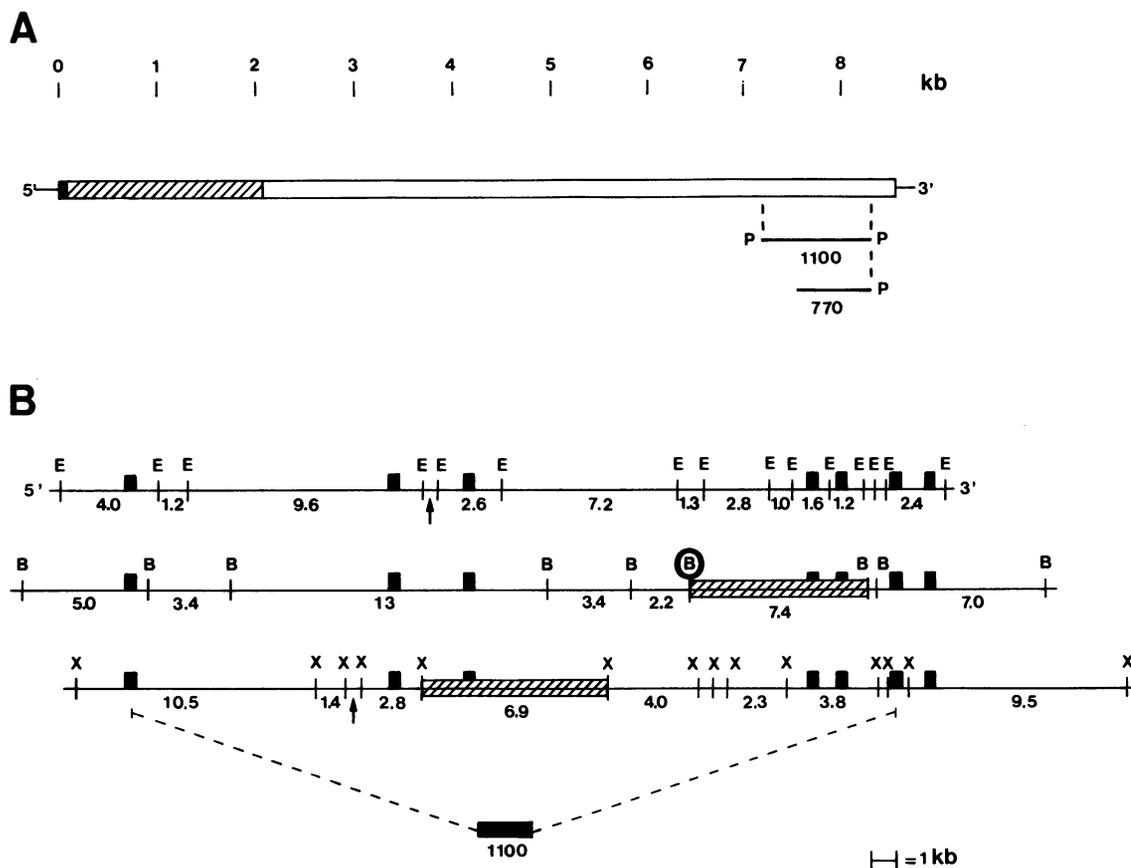
**DNA analysis.** Nuclear DNA was extracted from leukocytes after lysis of the erythrocytes as described (25, 26). 5–10 µg of DNA was digested to completion with either Bgl II or Xba I. Electrophoresis on a 0.8% agarose gel, transfer of DNA fragments to GeneScreen Plus membrane filters (New England Nuclear, Boston MA), and hybridization of genomic DNA fragments with probes that were labeled with <sup>32</sup>P by "nick translation," were done as described (27, 28). The filters were washed at a stringency of 0.3× SSC, 0.1% SDS at 65°C and autoradiography was performed. The probes employed were an approximately 1,100-bp Pst I vWF-cDNA fragment derived from pvWF2280 and an approximately 770-bp Pst I vWF-cDNA fragment derived from pvWF1210 (8). The 770-bp fragment is a part of the 1,100-bp fragment (Fig. 1 A). One of the two Pst I sites enclosing the 770-bp fragment is

derived from the polylinker of the vector pUC9. Digestion of chromosomal DNA with Bgl II yields two variant DNA fragments of 9 kb (A) and 7.4 kb (a), respectively (11), whereas digestion with Xba I results in variant fragments of 6.9 kb (B) and 5.2 kb (b), respectively (16).

**Isolation and characterization of genomic sequences.** Cosmid libraries were constructed with the double *cos* site vector C2RB (29) as described (30). The libraries were screened at a density of 50,000 colonies per dish (diameter 9 cm) (31). An 1,100-bp vWF-cDNA fragment was employed as the probe (14). Cosmid DNA was isolated according to a standard procedure (28). Mapping of restriction sites of the vWF-gene segment, spanned by the 1100-bp Pst I vWF-cDNA fragment, was performed on human vWF-genomic clones hybridizing with that particular vWF-cDNA fragment. The determination of the restriction sites was performed by standard procedures using single and double restriction-enzyme digestions in combination with Southern blot analysis with vWF-cDNA and vWF-genomic fragments as probes.

## Results

**Polymorphisms and chromosomal localization.** We have previously described two polymorphic sites in the vWF locus. First, a Bgl II polymorphism has been detected that has been identified using an 1,100-bp Pst I vWF-cDNA fragment derived from the 3' part of vWF cDNA (14). The frequency of



**Figure 1.** (A) Localization of the vWF-cDNA probes in full-length vWF cDNA. The full-length vWF cDNA is depicted: the coding sequence is indicated by a bar (solid bar, signal peptide; hatched bar, von Willebrand antigen II; open bar, mature vWF). The two probes used for RFLP analysis, the 770- and 1,100-bp fragments, are positioned beneath the corresponding part of the cDNA. (B) Restriction map of the vWF-gene segment, spanned by the 1,100-bp Pst I vWF-cDNA fragment. Each horizontal line illustrates the restriction map

of a particular restriction enzyme. The variant Xba I and Bgl II fragments are shown by hatched bars. The order of the 0.6- (see arrow) and 2.8-kb Xba I fragments and the 0.6- (see arrow) and 2.6-kb Eco RI fragments, respectively, is arbitrary. The fragments that hybridize with vWF cDNA are marked by a solid block, representing the presence of an exon (s) within that fragment. Restriction sites are abbreviated: B, Bgl II; E, Eco RI; P, Pst I, and X, Xba I. The inferred polymorphic Bgl II site is circled.

occurrence of the observed two variant restriction fragments of 9 kb and 7.4 kb is 0.69 and 0.31, respectively. Second, a Xba I polymorphism has been detected with a 770-bp Pst I fragment that had been isolated from the vWF-cDNA insert of pvWF1210 corresponding with the 3' part of vWF cDNA (16). Two variant Xba I fragments of ~ 6.9 and 5.2 kb occur at a frequency of 0.87 and 0.13, respectively.

To determine the location of the polymorphic Bgl II and Xba I sites we constructed a restriction map for both enzymes of that part of the vWF gene, spanned by the applied vWF-cDNA probes. For that purpose, two independent human cosmid libraries were screened with radiolabeled vWF-cDNA probes that comprised full-length vWF cDNA. Five cosmids were selected that contained overlapping vWF gene segments. The cosmid clones, which hybridize with the 1100-bp fragment, were used to construct the restriction map shown in Fig. 1 B. Southern blot analysis of the digested cosmid DNA revealed relatively weak signals for the 2.4-kb Eco RI-, the 7.0-kb Bgl II-, and the small 3' Xba I fragments (data not shown). This observation indicates the presence of an exon sequence in these fragments which covers a minor part of the 1,100-bp vWF cDNA. The lack of Eco RI, Bgl II, and Xba I sites within the 1,100-bp Pst I cDNA fragment implies the existence of at least five introns in the corresponding part of the vWF gene. Furthermore, this segment contains the polymorphic 6.9-kb Xba I and 7.4-kb Bgl II fragments (Fig. 1). The 7.4-kb Bgl II fragment is adjacent to a 2.2-kb Bgl II fragment. A mutation in the Bgl II site between these two fragments will yield a 9.6-kb fragment whose size corresponds with that of the other variant band detected with the 1,100-bp probe. Hence, we conclude that the polymorphic Bgl II site is located between the 2.2- and 7.4-kb fragments. A straightforward explanation for the occurrence of variant Xba I fragments of 6.9 and 5.2 kb is that a single mutation within the 6.9-kb Xba I fragment would create another Xba I site, resulting in an allele harboring a 5.2-kb fragment that hybridizes with the 1,100-bp probe.

*Genetic linkage between the RFLPs and vWD type IIA.* To ascertain whether the vWD type IIA is caused by a mutation within the vWF gene or by a defect in a factor involved in the vWF processing, we have investigated the linkage of vWD type IIA with the two intragenic polymorphic sites. For this reason, we have performed an analysis of the segregation of both these

polymorphisms and the vWD type IIA in a large family affected by this type of vWD. The pedigree of this family is depicted in Fig. 2. Bleeding times and plasma levels of vWF-Ag and vWFRco activity of the 39 family members were determined. The values of these parameters are listed in Table I. Based on the prolonged bleeding time, the reduction of vWFRco activity, and the nearly normal or decreased level of vWF Ag in plasma, these patients are diagnosed as vWD type IIA. Moreover, an analysis of the multimeric structure found in the plasma vWF of some of these patients shows that they lack the largest and intermediate-sized multimers. Furthermore, we observe that the minor bands of each multimer subset of type-IIA patients is overrepresented, a property which is characteristic for plasma vWF from vWD type-IIA patients (24) (Fig. 3).

DNA isolated from leukocytes of 39 members of this family was analyzed with both the Bgl II RFLP and the Xba I RFLP (Figs. 2 and 4). It should be noted that this family is informative for both polymorphisms. In the second generation, the affected siblings II2 and II3 are both heterozygous (Bb) for the Xba I RFLP, whereas II2 and II3 are homozygous (aa) and heterozygous (Aa) for the Bgl II RFLP, respectively. The genotype of the affected member II3 could be deduced from that of his children and must have been Aa and Bb. The affected children of both II2 and II3 all carry the a and b allele. The same observation is made for their affected grandchildren. Thus, both the a and b allele segregate with the mutation, causing the vWD type IIA. The significance of the linkage is further demonstrated by a LOD-score of 5.88. From this finding, we conclude that vWD type IIA is most likely due to a mutation within the vWF gene.

## Discussion

Three RFLPs for the enzymes Bgl II (13), Bam HI (15), and Xba I (16) have been described within the vWF gene. These RFLPs are observed in the normal population and, as shown in this paper, can be used as a genetic marker for segregation studies in families affected with vWD. Other RFLPs are anticipated to arise from the combination of different vWF-cDNA probes (also derived from full-length vWF cDNA [6-12]) with a panel of restriction enzymes. This development will increase

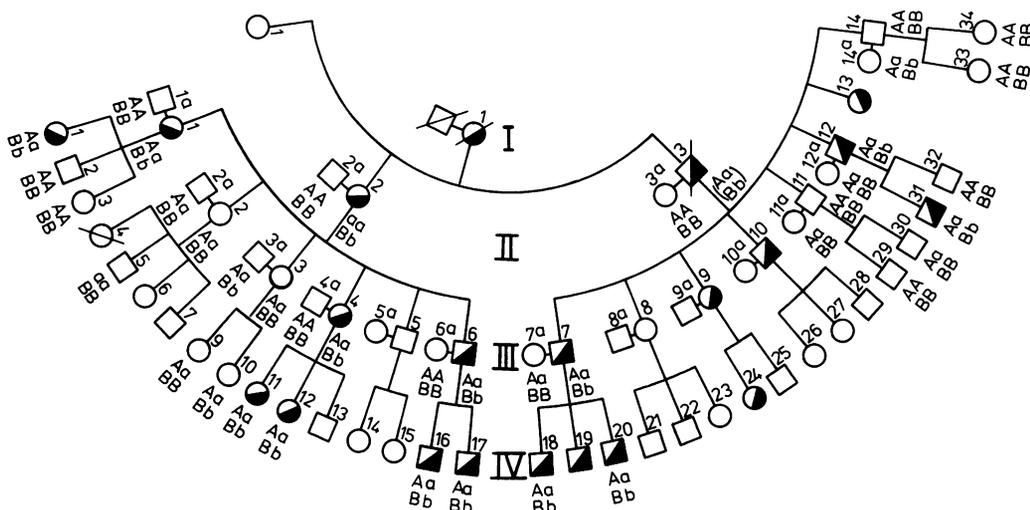


Figure 2. Pedigree of the family with vWD type IIA. In this family, the vWD trait is associated with the 7.4-kb Bgl II fragment (a) and the 5.2-kb Xba I fragment (b). □ and ○, male and female vWD family members, respectively. Half-filled symbols indicate individuals affected with vWD type IIA.

Table I. Results of the Phenotypic Analysis of the Affected Members of the Family with vWD

Patient pedigree number	vWF Ag	vWFRco	Bleeding time
II2	96	13	9 min 10 s
III1	94	15	>15 min
III4	102	13	5 min 44 s
III6	47	<10	>15 min
III7	47	<10	>15 min
III12	35	<10	7 min 30 s
IV1	59	<10	>15 min
IV11	36	<10	5 min 45 s
IV12	33	<10	>15 min
IV16	53	21	1 min 25 s
IV17	22	<10	6 min 55 s
IV18	37	<10	>15 min
IV20	25	<10	9 min 07 s
IV31	49	<10	>15 min

Both vWF-Ag and vWFRco activity are depicted as the percentage of normal. Determination of these parameters and the bleeding time was done as described in Methods.

the probability of encountering an allelic informativity in a family of interest. Such informative RFLPs will be of particular value for prenatal diagnosis of severe double heterozygous and homozygous forms of vWD. Besides the genetic alterations mentioned above, a number of nucleotide substitutions within exons of the vWF gene of presumably normal individuals are present. These differences are revealed by comparing the nucleotide sequence of independent vWF cDNAs (6–12). In three cases, a point mutation results in an amino-acid substitution (10). One of these alterations, an alanine vs. threonine substitution at position 26, has been discovered during an investigation of the amino-acid sequence of the mature vWF subunit (32). Apparently, this substitution does not affect the biological activity of the vWF protein.

Recently, new insight has been gained on the structure of vWF in the plasma of vWD type-IIA patients by Gralnick and co-workers (33). These authors show that the multimeric structure of vWF in the plasma of these patients is similar to that of healthy individuals, provided that blood is collected in a mixture of protease inhibitors. Although the multimeric structure appeared to be normal under these conditions, the

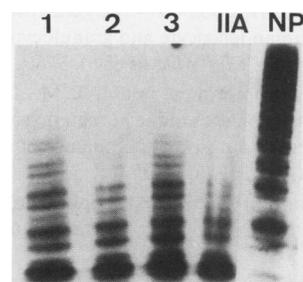


Figure 3. Agarose gel electrophoresis of plasma proteins and in situ immunostaining with <sup>125</sup>I-polyclonal anti-vWF IgG. Plasma samples were subjected to electrophoresis on a horizontal 1.4% agarose gel as described (21), and multimeric vWF was visualized by incubation of the gel with radiolabeled anti-vWF IgG. Lanes 1–3, plasma from affected members of the vWD type-IIA family (lane 1, III4; lane 2, III6; lane 3, III7); IIA, plasma from an established vWD type-IIA patient as a reference; NP, normal plasma.

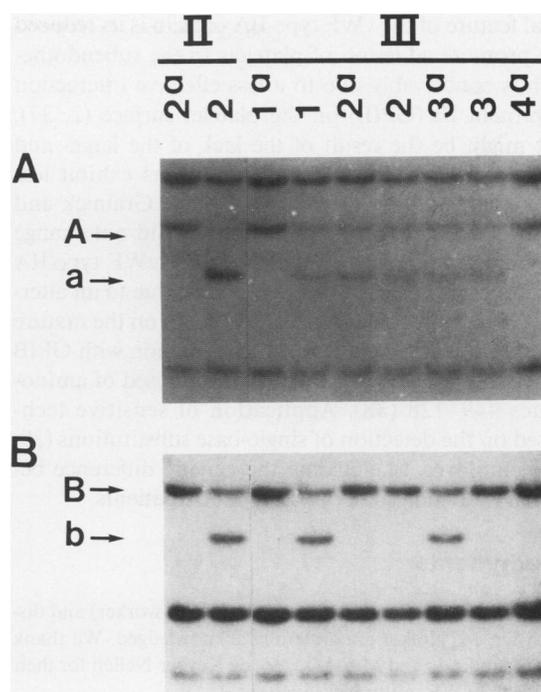


Figure 4. Southern blot analysis of both the Bgl II and Xba I RFLPs in a family affected with vWD type IIA. The DNA was digested with Bgl II (A) and Xba I (B) and hybridized with the radiolabeled 1,100- and 770-bp fragments, respectively. Results of the analysis of a number of family members are shown. The numbering of each individual corresponds with that of the pedigree. A, 9-kb Bgl II fragment; a, 7.4-kb Bgl II fragment; B, 6.9-kb Xba I fragment; b, 5.2-kb Xba I fragment.

vWFRco activity did not change significantly. Apparently, low vWFRco activity in these type-IIA patients is not due to an absence of the large- and intermediate-sized multimers, but rather to a functional defect in all polymers. This defect will be evoked either by an alteration of the primary amino-acid sequence in half of the subunits constituting the vWF multimers, or an abnormal posttranslational modification of the vWF protein. The latter possibility may be due to either a mutation in the vWF protein or it may be related to a mutation in a gene other than the vWF gene. However, we have to bear in mind that the vWD type IIA is a heterogeneous condition. For instance, the findings reported by Gralnick et al. have not been confirmed in all vWD type-IIA patients (34). Consequently, mechanisms responsible for the abnormalities of vWF IIA molecules may vary among different patients.

Our data demonstrate complete linkage of the genetic defect that caused vWD type IIA in the family we have studied with polymorphic sites located within the vWF gene. These observations strongly imply that the functional defect in the vWF protein of vWD type-IIA patients of this family is due to a mutation within one of the two vWF genes. This mutation results in an altered primary amino-acid sequence of the vWF subunit expressed by the defective gene. Hence, our interpretation excludes a defect in a factor involved in the processing of those type-IIA vWF molecules as a possible cause of the disease, as has been speculated by Wagner et al. (35, 36). That possibility is only valid if this factor is encoded by a gene in the direct vicinity of the vWF gene. Although difficult to exclude formally, this model is unnecessarily complex in light of the evidence presented above.

A typical feature of the vWF type-IIA protein is its reduced capacity to promote adhesion of platelets to the subendothelium, which is conceivably due to a less effective interaction with glycoprotein IB (GPIB) on the platelet surface (2, 37). This defect might be the result of the lack of the large- and intermediate-sized multimers. Small multimers exhibit less GPIB binding activity (2). Alternatively, since Gralnick and co-workers showed that the vWFRco activity did not change significantly upon correction of the abnormal vWF type-IIA multimeric structure (33), the defect might be due to an alteration in the GPIB binding domain. The domain on the mature vWF subunit that is responsible for the interaction with GPIB has been localized on a tryptic fragment composed of amino-acid residues 449–728 (38). Application of sensitive techniques, based on the detection of single-base substitutions (39, 40), can be employed to elucidate the genetic difference between normal individuals and vWD type-IIA patients.

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