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(71) Applicant: **BAXTER INTERNATIONAL INC. ONE BAXTER PARKWAY, DEERFIELD, IL 60015 IN US**
BAXTER HEALTHCARE S.A THURGAUERSTRASSES 130, CH-8152 GLATTPARK CH

(72) Inventor: **LAI, CHEE KONG 1 SILVER BIRCH LANE LITTLETON, MA 01460 US**
STAFFORD, RODDY, KEVIN 234 SPRING STREET SHREWSBURY, MA 01545 US

(54) **Title:**
A NEW VARIANT OF ANTIHEMOPHILIC FACTOR VIII HAVING INCREASED SPECIFIC ACTIVITY

(57) **Abstract:**
The present invention is in the field of hemophilia therapy. It relates to a new variant of antihemophilic factor VIII having increased specific activity in comparison to known factor VIII products.

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(71) Applicant *(for all designated States except US)*: **IPSEN PHARMA S.A.S.** [FR/FR]; 65, Quai Georges Gorse, F-92100 Boulogne-billancourt (FR).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **LAI, Chee Kong** [MY/US]; 1 Silver Birch Lane, Littleton, MA 01460 (US). **STAFFORD, Roddy, Kevin** [US/US]; 234 Spring Street, Shrewsbury, MA 01545 (US).

(74) Agents: **FEENEY, Alan, F.** et al.; Biomeasure, Incorporated, 27 Maple Street, Milford, Massachusetts 01757-3650 (US).

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(54) Title: A NEW VARIANT OF ANTIHEMOPHILIC FACTOR VIII HAVING INCREASED SPECIFIC ACTIVITY

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A new variant of antihemophilic factor VIII having increased specific activity

FIELD OF THE INVENTION

The present invention is in the field of blood coagulation factors and hemophilia. It relates to a new variant of antihemophilic factor VIII (FVIII), designated herein rFVIIIv3, having an increased specific activity in comparison to known factor VIII products.

BACKGROUND OF THE INVENTION

Blood clotting begins when platelets adhere to the cut wall of an injured blood vessel at a lesion site. Subsequently, in a cascade of enzymatically regulated reactions, soluble fibrinogen molecules are converted by the enzyme thrombin to insoluble strands of fibrin that hold the platelets together in a thrombus. At each step in the cascade, a variant precursor is converted to a protease that cleaves the next variant precursor in the series. Cofactors are required at most of the steps.

Factor VIII (also called antihemophilic factor VIII or FVIII) circulates as an inactive precursor in blood, bound tightly and non-covalently to von Willebrand factor. Factor VIII is proteolytically activated by thrombin or factor Xa, which dissociates it from von Willebrand factor and activates its procoagulant function in the cascade. In its active form, the variant factor VIIIa is a cofactor that increases the catalytic efficiency of factor IXa toward factor X activation by several orders of magnitude.

Cloning of human FVIII has revealed that the variant contains 2332 amino acids organized within a number of domains with the sequence A1-A2-B-A3-C1-C2 (Vehar et al., 1984, Nature 312, 337-342; Toole et al., 1984, Nature 312, 342-347 and Wood et al., 1984, Nature 312, 330-337). Most of FVIII is heterodimeric in plasma, containing a light-chain and various heavy-chain derivatives. The heterodimeric structure is due to proteolytic cleavage of the precursor at arginine^{1648}, resulting in heavy- and light-chains comprising A_1-A_2-B and A_3-C_1-C_2, respectively. The heterogeneity within the heavy chain is explained by limited proteolysis within its carboxy-terminal B-domain.

In order to function as a co-factor for factor X activation, FVIII requires limited proteolysis by factor Xa or thrombin. This activation involves cleavage at arginine at positions 372 and 740 on the heavy-chain and at position 1689 on the light-chain. It has been established that in comparison with the inactive precursor, active FVIII

cofactor lacks a light chain fragment 1649-1689 and the whole B-domain (Mertens *et al.,* 1993).

People with deficiencies in factor VIII or antibodies against factor VIII who are not treated with factor VIII suffer uncontrolled internal bleeding that may cause a range of serious symptoms, from inflammatory reactions in joints to early death. Severe hemophiliacs, who number about 10,000 in the United States, can be treated with infusion of human factor VIII, which will restore the blood's normal clotting ability if administered with sufficient frequency and concentration. The classic definition of factor VIII, in fact, is the substance present in normal blood plasma that corrects the clotting defect in plasma derived from individuals with hemophilia A.

Several preparations of human plasma-derived factor VIII of varying degrees of purity are available commercially for the treatment of hemophilia A. These include a partially purified factor VIII derived from the pooled blood of many donors that is heat- and detergent treated for viruses but contain a significant level of antigenic variants; a monoclonal antibody purified factor VIII that has lower levels of antigenic impurities and viral contamination; and recombinant human factor VIII.

The development of antibodies ("inhibitors" or "inhibitory antibodies") that inhibit the activity of factor VIII is a serious complication in the management of patients with hemophilia.

Alloantibodies develop in approximately 20% of patients with hemophilia A in response to therapeutic infusions of factor VIII. In previously untreated patients with hemophilia A who develop inhibitors, the inhibitor usually develops within one year of treatment. Additionally, antibodies (autoantibodies) that inactivate factor VIII occasionally develop in individuals with previously normal factor VIII levels. If the inhibitor titer is low enough, patients can be managed by increasing the dose of factor VIII. However, often the inhibitor titer is so high that it cannot be overwhelmed by factor VIII. An alternative strategy is to bypass the need for factor VIII during normal hemostasis using activated prothrombin complex concentrate preparations (for example, KONYNE (Cutter Laboratories), FEIBA (Baxter Healthcare), PROPLEX (Baxter Healthcare)) or recombinant human factor VIIa. Additionally, since porcine factor VIII usually has substantially less reactivity with inhibitors than human factor VIII, a partially purified porcine factor VIII preparation (HYATE: C (IPSEN Pharma)) has been used. Many patients who have developed inhibitory antibodies to human factor VIII have been successfully treated with porcine factor VIII and have tolerated such treatment for long periods of time.

However, public health concerns regarding the risk of viruses or other blood-borne contaminants have limited the usefulness of porcine factor VIII purified from porcine blood. A recombinant porcine factor VIII variant has therefore been developed, which is designated "OBI-1" and described e.g. in WO 01/68109. OBI-1 is a partially B-domain deleted porcine FVIII. This molecule is presently in clinical development.

B-domain deleted factor VIII variants are known to keep the procoagulant and cofactor activity of factor VIII. In addition to this, Mertens et al. (British Journal of Haematology 1993, 85, 133-142) describe recombinant human factor VIII variants lacking the B-domain and the heavy-chain sequence spanning from Lysine 713 to Arginine 740.

Many hemophiliacs require daily replacement of factor VIII to prevent bleeding and the resulting deforming hemophilic arthropathy. In view of this, there is a need for a more potent factor VIII molecule.

SUMMARY OF THE INVENTION

In a first aspect, the invention relates to an isolated, recombinant, fully or partially B-domain deleted factor VIII (FVIII) variant, the FVIII variant being devoid of an up to 27 amino acid sequence corresponding to amino acids 716 to 742 of porcine factor VIII as depicted in Figure 2 or amino acids 714 to 740 of SEQ ID NO: 7. This 27 amino acid sequence, NTGDYYEDSYEDISAYLLSKNNAIEPR (SEQ ID NO:10), may be partially or completely deleted.

In one embodiment, the invention relates to an isolated, recombinant, fully or partially B-domain deleted FVIII variant, the FVIII variant being devoid of an up to 27 amino acid sequence corresponding to amino acids 716 to 742 of porcine factor VIII as depicted in Figure 2 or amino acids 714 to 740 of SEQ ID NO: 7, where 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acids may be deleted. In another embodiment, 27, 26, 25, 24, 23, 22, 21 or 20 amino acids are deleted. In another embodiment, 27, 26, or 25 amino acids are deleted. In another embodiment, the 27 amino acids corresponding to DIGDYYDNTYEDIPGFLLSGKNVIEPR (SEQ ID NO:10) are deleted.

In one embodiment, the invention relates to an isolated, recombinant, fully or partially B-domain deleted FVIII variant, the FVIII variant being devoid of an up to 27 amino acid sequence corresponding to amino acids 716 to 742 of human factor VIII as depicted in Figure 2 or amino acids 714 to 740 of SEQ ID NO: 8, where 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acids may be deleted. In another embodiment, 27, 26, 25, 24, 23, 22, 21 or

20 amino acids are deleted. In another embodiment, 27, 26, or 25 amino acids are deleted. In another embodiment, the 27 amino acids corresponding to NTGDYYEDSYEDISAYLLSKNNAIEPR (SEQ ID NO:11) are deleted.

In another embodiment, the invention relates to an isolated, recombinant, fully or partially B-domain deleted porcine FVIII variant being completely devoid of the 27 amino acids corresponding to DIGDYYDNTYEDIPGFLLSGKNVIEPR (SEQ ID NO:10).

In another embodiment, the invention relates to an isolated, recombinant, fully or partially B-domain deleted human FVIII variant being completely devoid of the 27 amino acids corresponding to NTGDYYEDSYEDISAYLLSKNNAIEPR (SEQ ID NO:11).

In another embodiment, the invention relates to an isolated, recombinant, fully or partially B-domain deleted canine FVIII variant being completely devoid of the 27 amino acids corresponding to NIDDYYEDTYEDIPTPLLNENNVIKPR (SEQ ID NO:12).

A second aspect of the invention relates to a polynucleotide encoding a polypeptide as defined in the first aspect and the embodiments described.

In a third aspect, the invention relates to an expression vector comprising a polynucleotide as defined in the second aspect.

A fourth aspect of the invention relates to a mammalian cell comprising an expression vector as defined in the third aspect.

In a fifth aspect, the invention relates to a method for producing a FVIII as defined in the first aspect, comprising the steps of:

a. Culturing a mammalian cell according to the fourth aspect; and

b. Isolating from the mammalian cell the FVIII variant.

A sixth aspect of the invention relates to a pharmaceutical composition comprising a FVIII variant as defined in the first aspect.

In a seventh aspect, the invention relates to a method of treating a patient suffering from hemophilia comprising administering a therapeutically effective amount of FVIII variant according to the first aspect of the invention to a patient in need thereof, thereby treating the hemophilia in said patient.

In an eighth aspect, the invention relates to a FVIII variant according to the first aspect for use in treating hemophilia.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the amount of protein in µg per dose required for 5 known FVIII products (blank columns) and the new rpFVIIIv3 (hashed column). BDD=B-domain deleted; FL=full length; u=µ;

Fig. 2 a-e are continuations of the same sequence. Fig 2a-e shows a sequence alignment between the factor VIII sequences from human (homo sapiens), pig (sus scrofa), mouse (mus musculus) and dog (canis familiaris). This sequence alignment is taken from HADB (aka HAMSTeRS) 2010, home page of the Haemophilia A Mutation Database and a resource site for work on factor VIII (hadb.org.uk). The numbering follows the human sequence and is not identical to the amino acid numbers of the sequences in the Sequence Listing. The bold plus double underlined sequence depicts the sequence missing in the recombinant FVIIIv3 variants of the invention. The underlined sequence is the sequence of the B-domain. The sequence highlighted in grey is the portion from the B-domain which can be retained in the partially B-domain deleted FIIIv3 sequences of the invention; and

Fig. 3 shows typical example of the SEC profile of the calibration standards and the linear regression of the calibration curve as described in Example 5.

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO: 1 shows the sequence of porcine partially B-domain deleted FVIIIv3;

SEQ ID NO: 2 shows the sequence of a human B-domain deleted FVIIIv3;

SEQ ID NO: 3 shows the sequence of a canine B-domain deleted FVIIIv3;

SEQ ID NO: 4 shows the sequence from the porcine FVIII B-domain that can be present in a partially B-domain deleted FVIII molecule of the invention (the so called “B-domain linker”);

SEQ ID NO: 5 shows a B-domain linker sequence from the human FVIII B-domain that can be present in a partially B-domain deleted FVIII molecule of the invention;

SEQ ID NO: 6 shows a B-domain linker sequence from the canine FVIII B-domain that can be present in a partially B-domain deleted FVIII molecule of the invention;

SEQ ID NO: 7 shows the amino acid sequence of full-length porcine FVIII;

SEQ ID NO: 8 shows the amino acid sequence of full-length human FVIII;

SEQ ID NO: 9 shows the amino acid sequence of full-length canine FVIII;

SEQ ID NO:10 shows the 27 peptide sequence which corresponds to amino acids 716 to 742 of porcine factor VIII as depicted in Figure 2 or amino acids 714 to 740 of SEQ ID NO: 7;

SEQ ID NO:11 shows the 27 peptide sequence which corresponds to amino acids 714 to 740 of human factor VIII as depicted in Figure 2 or amino acids 714 to 740 of SEQ ID NO: 8; and

SEQ ID NO:12 shows the 27 peptide sequence which corresponds to amino acids 714 to 740 of canine factor VIII as depicted in Figure 2 or amino acids 708 to 734 of SEQ ID NO: 9.

DETAILED DESCRIPTION OF THE INVENTION

The invention is based on the finding of a variant of a partially B-domain deleted recombinant porcine factor VIII protein which contains a particular 27 amino acid deletion. This variant, which is herein designated rpFVIIIv3, has been shown to have an increased specific activity as compared to a similar protein containing the 27 amino acid stretch, namely a partially B-domain deleted recombinant porcine FVIII called “OBI-1”. OBI-1, its amino acid sequence and polynucleotides encoding OBI-1 are known e.g. from US 6,458,563.

The invention relates to an isolated, recombinant, fully or partially B-domain deleted factor VIII (FVIII) variant, wherein the FVIII variant is devoid of an amino acid sequence corresponding to amino acids 716 to 742 of porcine factor VIII as depicted in Figure 2 or amino acids 714 to 740 of SEQ ID NO: 7.

Additionally, the invention relates to an isolated, recombinant, fully or partially B-domain deleted FVIII variant, the FVIII variant being devoid of a 27 amino acid sequence corresponding to amino acids 716 to 742 of porcine factor VIII as depicted in Figure 2 or amino acids 714 to 740 of SEQ ID NO: 7, where 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acids may be deleted. In one variant, 27, 26, 25, 24, 23, 22, 21 or 20 amino acids are deleted. In another variant, 27, 26, or 25 amino acids are deleted. In the rpFVIIIv3 variant, the 27 amino acids corresponding to DIGDYYDNTYEDIPGFLLSGKNVIEPR (SEQ ID NO:10) are deleted.

Additionally, the invention relates to an isolated, recombinant, fully or partially B-domain deleted FVIII variant, the FVIII variant being devoid of a 27 amino acid sequence corresponding to amino acids 714 to 740 of human factor VIII as depicted

in Figure 2 or amino acids 714 to 740 of SEQ ID NO: 8, where 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acids may be deleted. In one variant, 27, 26, 25, 24, 23, 22, 21 or 20 amino acids are deleted. In another variant, 27, 26, or 25 amino acids are deleted. In a human FVIIIv3 variant (rhFVIIIv3), the 27 amino acids corresponding to NTGDYYEDSYEDISAYLLSKNNAIEPR (SEQ ID NO:11) are deleted.

Additionally, the invention relates to an isolated, recombinant, fully or partially B-domain deleted FVIII variant, the FVIII variant being devoid of a 27 amino acid sequence corresponding to amino acids 714 to 740 of canine factor VIII as depicted in Figure 2 or amino acids 708 to 734 of SEQ ID NO: 9, where 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acids may be deleted. In one variant, 27, 26, 25, 24, 23, 22, 21 or 20 amino acids are deleted. In another variant, 27, 26, or 25 amino acids are deleted. In a canine FVIIIv3 variant (rcFVIIIv3), the 27 amino acids corresponding to NIDDYYEDTYEDIPTPLLNENNVIKPR (SEQ ID NO:12) are deleted.

Such variants have an elevated specific activity as compared to similar variants containing the intact 27 amino acid sequence. Preferably, the specific activity is increased by more than 25 % or 30 % or 35 % or 40 % or 45 % or 50 % or 55 % or 60 %.

The term “specific activity," as used herein, refers to the activity that will correct the coagulation defect of human factor VIII deficient plasma. Specific activity is measured in units of clotting activity per milligram total factor VIII variant in a standard assay in which the clotting time of human factor VIII deficient plasma is compared to that of normal human plasma. A suitable standard assay for measuring the potency of FVIII products, which is accepted by the FDA for high purity FVIII concentrates, is called the one-stage clotting assay (OSCA; see Example 5; Langdell RD, Wagner RH, Brinkhous KM., Effect of antihemophilic factor on one-stage clotting tests: a presumptive test for hemophilia and a simple one-stage antihemophilic assay procedure, J Lab Clin Med, 1953; 41:637–47; Brand JT, Measurement of factor VIII: a potential risk factor for vascular disease, Arch Pathol Lab Med, 1993; 117:48–51; Preston FE, Kitchen S, Quality control and factor VIII assays, Haemophilia, 1998; 4:651–3; National Committee for Clinical Laboratory Standards (NCCLS USA). Determination of factor coagulant activities; Approved guideline, NCCLS Document H-48-A 1997; 17:1–36.). The amount of FVIII protein present in a sample can be measured *e.g.* by SEC HPLC (size-exclusion high-performance liquid chromatography).

One unit of factor VIII activity is the activity present in one milliliter of normal human plasma. In the assay, the shorter the time for clot formation, the greater the activity of the factor VIII being assayed. Porcine factor VIII has coagulation activity in a human factor VIII assay.

In line with the present invention, the terms "protein" and "polypeptide" are being used interchangeably.

Being "devoid of an amino acid sequence corresponding to amino acids 716 to 742 as depicted in Fig. 2" means that amino acids 716 and 742, as well as the amino acids between these positions, are deleted, *i.e.* not included, in the FVIII variant of the invention. This stretch of amino acids consists thus of 27 amino acids, of which the FVIII variants of the invention are devoid.

The factor VIII variants of the invention are herein globally designated "FVIII variant(s)" or "FVIIIv3" or "rFVIIIv3". They can be of porcine, human, canine, murine or any other origin which is appropriate for human or animal therapy. Fig. 2 depicts a sequence alignment of FVIII sequences of human, porcine, murine and canine origin.

The sequence deleted in the FVIII variants of the invention is highlighted in bold and double underlined. This stretch of amino acids can be identified for the FVIII variants of other species by the person skilled in the art by aligning the FVIII sequences of further species with *e.g.* the porcine or human sequence and taking the amino acids that correspond to amino acids 716 to 742 as per Fig. 2 of the present patent application.

Embodiments of the invention relate to FVIII variants of porcine, human or canine origin.

In an embodiment, the FVIII variant according to the invention comprises the sequence of SEQ ID NO:1, or a variant thereof comprising a sequence being at least 90% identical to SEQ ID NO: 1. It is understood that the 10% variability concerns the sequences outside of the deleted stretch of amino acids, *i.e.* that the variant is completely devoid of DIGDYYDNTYEDIPGFLLSGKNVIEPR (SEQ ID NO:10), an amino acid sequence corresponding to amino acids 716 to 742 of porcine factor VIII as depicted in Figure 2 or amino acids 714 to 740 of SEQ ID NO: 7.

In a further embodiment, the FVIII variant of the invention comprises the sequence of SEQ ID NO:2, or a variant thereof comprising a sequence being at least 90% identical to SEQ ID NO: 2. Again, the variant is understood to be completely devoid of an amino acid sequence NTGDYYEDSYEDISAYLLSKNNAIEPR (SEQ ID NO:11),

corresponding to amino acids 714 to 740 of human factor VIII as depicted in Fig. 2 or amino acids 714 to 740 of SEQ ID NO: 8.

In yet a further embodiment, the FVIII variant of the invention comprises the sequence of SEQ ID NO:3, or a variant thereof comprising a sequence being at least 90% identical to SEQ ID NO: 3. Again, the variant is understood to be completely devoid of an amino acid sequence NIDDYYEDTYEDIPTPLLNENNVIKPR (SEQ ID NO:12) corresponding to amino acids 714 to 740 of canine factor VIII as depicted in Fig. 2 or amino acids 708 to 734 of SEQ ID NO: 9.

The FVIII variants of the invention can also comprise sequences that are at least 91, 92, 93, 94, 95, 96, 97, 98, 99, 99.5, 99.6, 99.7, 99.8 or 99.9% identical to the sequences of SEQ ID NO: 7, 8 or 9.

To determine the percent identity of two polypeptides/proteins, the amino acid sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid sequence for optimal alignment with a second amino acid sequence). The amino acid residues at corresponding amino acid positions are then compared. When a position in the first sequence is occupied by the same amino acid residue as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.,* % identity=# of identical positions/total # of positions (*e.g.,* (overlapping positions)×100).

The determination of percent identity between two sequences can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul (1990) Proc. Natl. Acad. Sci. USA 87:2264–2268, modified as in Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873–5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, *et al.* (1990) J. Mol. Biol. 215:403–410. BLAST nucleotide searches can be performed with the NBLAST program, score=100, wordlength=12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the invention. BLAST variant searches can be performed with the XBLAST program, score=50, wordlength=3 to obtain amino acid sequences homologous to a variant molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) Nucleic Acids Res. 25:3389–3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant

relationships between molecules. When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (*e.g.,* XBLAST and NBLAST) can be used. Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, (1988) CABIOS 4:11–17. Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used. Yet another useful algorithm for identifying regions of local sequence similarity and alignment is the FASTA algorithm as described in Pearson and Lipman (1988) Proc. Natl. Acad. Sci. USA 85:2444–2448. When using the FASTA algorithm for comparing nucleotide or amino acid sequences, a PAM120 weight residue table can, for example, be used with a k-tuple value of 2.

The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, only exact matches are counted.

The FVIII variant of the invention can be partially or fully B-domain deleted. This means that either the whole B-domain is deleted, or a part of the B-domain is retained in the FVIII variant. The remaining (retained) part of the B-domain is e.g. selected from the 20, 15, 12, 10 or 5 N-terminal amino acids and the 20, 15, 12, 10 or 5 C-terminal amino acids of the B-domain, fused in frame with each other.

Hence, preferably, in the partially B-domain deleted FVIII variants of the invention, significant portions of the approximately 900 amino acid B-domain are being removed. In embodiments of the invention, less than 5% or less than 4% or less than 3% or less than 2 % or less than 1 % of the B-domain remain present in the FVIII variants.

In embodiments of the invention, the remaining portion of the B-domain is selected from a sequence consisting of SEQ ID NO: 4 or SEQ ID NO:5 or SEQ ID NO: 6.

In a further aspect, the invention relates to a polynucleotide encoding a polypeptide/protein as described above. A polynucleotide can be an RNA or DNA molecule whose nucleotide sequence embodies coding information to a host cell for the amino acid sequence of the variant of the invention, according to the known relationships of the genetic code.

In a further aspect, the invention relates to an expression vector comprising a polynucleotide as described above.

An "expression vector" is a DNA element, often of circular structure, having the ability to replicate autonomously in a desired host cell, or to integrate into a host cell genome and also possessing certain well-known features which permit expression of a coding DNA inserted into the vector sequence at the proper site and in proper orientation. Such features can include, but are not limited to, one or more promoter sequences to direct transcription initiation of the coding DNA and other DNA elements such as enhancers, polyadenylation sites and the like, all as well known in the art. The term "expression vector" is used to denote both a vector having a DNA coding sequence to be expressed inserted within its sequence, and a vector having the requisite expression control elements so arranged with respect to an insertion site that it can serve to express any coding DNA inserted into the site, all as well-known in the art. Thus, for example, a vector lacking a promoter can become an expression vector by the insertion of a promoter combined with a coding DNA. An expression vector, as used herein, can also be a viral vector.

Expression of the recombinant FVIII variants of the invention is preferably carried out in mammalian cell culture.

Therefore, in a further aspect, the invention relates to a mammalian cell comprising an expression vector as described above. For instance, CHO (Chinese hamster ovary) cells and BHK cells (baby hamster kidney cells) are mammalian cells that are suitable host cells in the context of the present invention.

In accordance with another aspect of the invention, a method for producing a FVIII variant of the invention comprises the steps of:

a. Culturing a mammalian cell as described above; and

b. Isolating from the mammalian cell the FVIII variant.

In an embodiment, the method further comprises the step of

c. Formulating the factor VIII variant together with appropriate excipients into a pharmaceutical composition.

Excipients suitable for human or animal administration are e.g. pharmaceutical stabilization compounds, preservatives, delivery vehicles, and/or carrier vehicles. One suitable formulation for factor VIII products is e.g. described in WO 03/080108, which is incorporated by reference herein.

In a further aspect, the invention relates to a pharmaceutical composition comprising a FVIII variant of the invention.

A further aspect of the invention relates to a method of treating a patient suffering from factor VIII deficiency comprising administering a therapeutically effective amount of FVIII variant of the invention to a patient in need thereof, thereby treating the factor VIII deficiency in said patient.

The term "therapeutically effective amount" as used herein, means the level of FVIII in the plasma of a patient having FVIII deficiency, who has received a pharmaceutical composition of FVIII variant, that is sufficient to exhibit a measurable improvement or protective effect in the patient (e.g., to stop bleeding). The patients having FVIII deficiency are typically congenital hemophilia A patients but also include those subjects diagnosed with "acquired hemophilia", a condition in which those who are not congenital hemophiliacs spontaneously develop inhibitory antibodies to their FVIII, creating a serious FVIII deficiency.

The invention also relates to a FVIII variant of the invention for use in treating factor VIII deficiency.

Treatment can take the form of a single intravenous administration of the composition or periodic or continuous administration over an extended period of time, as required. Preferably, administration of the FVIII variant is by intravenous route.

"Factor VIII deficiency," as used herein, includes deficiency in clotting activity caused by production of defective factor VIII, by inadequate or no production of factor VIII, or by partial or total inhibition of factor VIII by inhibitors. Hemophilia A is a type of factor VIII deficiency resulting from a defect in an X-linked gene and the absence or deficiency of the factor VIII variant it encodes.

The FVIII variants of the invention are used to treat uncontrolled bleeding due to factor VIII deficiency (*e.g.,* intraarticular, intracranial, or gastrointestinal hemorrhage) in hemophiliacs.

In an embodiment of the invention, the factor VIII deficiency is hemophilia A.

In another embodiment, the factor VIII deficiency is acquired hemophilia.

In an embodiment, the factor VIII deficiency is treated in patients having developed human FVIII antibodies.

As mentioned above, the FVIII variants of the invention have increased specific activity. It is therefore possible to administer reduced amounts of FVIII variant in order to treat VIII deficiency in accordance with the present invention. The amounts of FVIII variant can be reduced by at least 30%, 35%, 40%, 45%, 50%, 55%, 60% as

compared to therapy with a FVIII product that does not contain the up to 27 amino acid deletion of the invention.

Reducing the amount of FVIII variant therapy has the advantage of expected reduced immunogenicity, *i.e.* it is expected that the probability of generation of inhibitory antibodies against the FVIII replacement therapy in the patient is significantly reduced.

In an embodiment, a factor VIII variant of the invention is administered in an amount of no more than 200 µg/dose or 150 µg/dose or 145 µg/dose or 140 µg/dose or 136 µg/dose or 130 µg/dose.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations and conditions without departing from the spirit and scope of the invention and without undue experimentation.

While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.

All references cited herein, including journal articles or abstracts, published or unpublished U. S. or foreign patent application, issued U. S. or foreign patents or any other references, are entirely incorporated by reference herein, including all data, tables, figures and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by reference.

Reference to known method steps, conventional methods steps, known methods or conventional methods is not any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various application such specific embodiments, without undue experimentation,

without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning a range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

EXAMPLES

Example 1: Expression of recombinant porcine partially B-domain deleted FVIII (OBI-1) and isolation of three variant proteins (rpVIIIv1, v2 and v3)

Expression of OBI-1 in BHK cells was carried out essentially as described in US 6,458,563. Three variants were detected in the produced material (rpVIIIv1, v2 and v3). The variants were isolated and purified to >95% purity ion exchange chromatography. For purification, an AKTA Explorer system (AKTA Explorer 10, GE Healthcare Lifesciences # 17-5167-01) using a MonoQ HR10/10 semi-preparative anion exchange column (now Mono Q 10/100 GL ,GE Healthcare Lifesciences # 17-5167-01) was used. Elution was carried out on a gradient with the two following buffers:

Buffer A: 10 mM TRIS, pH 7.0, 0.01% Polysorbate 80

Buffer B: Buffer A and 1 M sodium chloride.

The purification method characteristics are summarized in Table 1.

Table 1: rpFVIII Variant Purification Method

AKTA Method:	LoopMQ1010	
Flow:	4 ml/min	
Equilibrium Column	8 CV	
Load flow	4ml/min	
Sample Volume	40 - 1000 ml	
Wash Column	10CV	
Gradient	Column Volume (CV)	%B
	7	45
	4	50
	4	50
	6	52
	4	52
	2	55
	7	60
	1	65

	5	65
	1	70
	5	70
	1	100
	7	100
	1	10
	4	10
Total Run	59 CV	
Column Temp	Not controlled	

Example 2: Variant 3 (rpVIIIv3) has a significantly higher specific activity than variants 1 and 2

The specific activity of each variant was assessed by dividing the potency assessed by one stage coagulation assay (OSCA) by the protein concentration as measured by the SEC HPLC per method. The OSCA and SEC HPLC methods were carried out as described in "Materials and Methods" Example 5.

RESULTS

The results for the specific activities are indicated in Table 2.

Table 2: Specific Activities (OSCA method)for Purified Variants 1, 2 and 3 obtained from two different lots of rpFVIII

Sample based on SEC	OSCA units/ml	%RSD*	Specific activity units/ml	%RSD*
V1 – lot 1	1060	1.5	11956	1.7
V2 – lot 1	715	2.7	17267	3.3
V3 – lot 1	216	1.2	25926	2.8
OBI-1	513	1	18150	1.3
V1 – lot 2	980	1.2	12532	1.5
V2 – lot 2	613	0.6	16089	1.3
V3 – lot 2	280	2.9	19718	4.1
OBI-1	565	4.0	14427	4.4

*relative standard deviation

The observation of significantly higher specific activity for variant 3 in the OSCA assay is distinct and consistent. Since the OSCA method is predictive and representative for the coagulation process in humans, it is expected that variant 3 is a product of significant value. It is expected that 1.5-2.0 fold increase in potency reduce the amount of FVIII administered to patients and might therefore indirectly reduce the occurrence of immunological side effects.

Example 3: Sequence determination of rpFVIIIv3

The sequence of purified v3 was determined by peptide map followed by LCMS (liquid chromatography mass spectrometry) and LCMS/MS (liquid chromatography mass spectrometry/mass spectrometry) on a Q-TOF Ultima Mass Spectrometer, running MassLynx 4.0 (Waters Corporation, Milford, MA).

Approximately 250 µg of Variant 3 was concentrated using an Amicon, Centricon YM-10 concentrator with a 10,000 MWCO filter (Millipore Corporation, Billerica, MA) to a volume of < 100 µL. The samples were mixed with 450 µL of 6 M guanidine HCl/ 0.002 M EDTA (ethylenediaminetetraacetic acid) /0.02 M Tris buffer pH 8 and transferred to 2.0 mL polypropylene tubes. 1 M DTT (dithiothreitol) was added to each sample to a final concentration of 10 mM and incubated for 1 hour at 37 °C. After reduction, 2 M iodoacetamide was added to each tube to a final concentration of 20 mM and incubated for an additional hour at room temperature. The reduced and alkylated samples were transferred to dialysis cassettes and dialyzed for 1 hour against 1 L of 50 mM ammonium bicarbonate dialysis buffer containing 1.0 M urea, pH 8. The samples were then dialyzed against 1 L of dialysis buffer overnight at room temperature while maintaining constant stirring. After dialysis the final volumes were approximately 0.5 mL each. Samples were divided into two 125 µg aliquots.

After dialysis, the protein sample was contained in a matrix that is optimal for proteolytic digestion with trypsin. Trypsin was added to each sample at an enzyme to substrate ratio of 1:20 (w:w) and incubated for 8 hours at 37 °C. All samples were transferred to HPLC vials and analyzed by HPLC-MS.

Reduced and alkylated protein was injected onto a Vydac C18 reverse phase column (Grace, Deerfield, IL). Prior to the sample injection, a blank sample (Mobile Phase A; deionized water containing 0.2% (v:v) formic acid) was analyzed to equilibrate the column and demonstrate the absence of interfering peaks.

Mass spectrometry data was collected on a Q-TOF API-US mass spectrometer or a Q-TOF Ultima mass spectrometer (Waters Corporation, Milford, MA) using electrospray ionization (ESI) in the positive ion mode. Prior to sample analysis, the mass spectrometer was calibrated using a 5th order fit on the fragment ions of Glu-Fibrinogen peptide covering a range of 175 to 1285 m/z. For the calibration to pass specifications, a RMS (root mean square) error for the mass of the peptide fragments less than 5 ppm was required. The software package Masslynx 4.0™ SP2 and SP4 (Waters Corporation, Milford, MA) were used for data acquisition and analysis.

Both MS and MS/MS data were collected using a single liquid chromatography (LC) run. Full mass spectrometer (MS) survey scans were collected from 200-1950 m/z.

The sequence of rpFVIII was determined to correspond to SEQ ID NO: 1. This sequence contains a 27 amino acid deletion in comparison with the OBI-1 sequence.

Example 4: rpFVIIIv3 has a higher specific activity than other known FVIII products

The specific activity of rpFVIIIv3 was compared to three commercially available FVIII products, namely Xynthia® (a recombinant, B-domain deleted human FVIII from Wyeth Pharmaceuticals (Philadelphia, PA)), Kogenate FS® (a full-length human FVIII from Bayer Healthcare (Tarrytown, NY)) and Advate® (a full-length human FVIII from Baxter (Westlake Village, CA)), as well as to OBI-1 (a recombinant partially B-domain deleted porcine FVIII), which is presently under clinical development, and a B-domain deleted canine factor VIII (Denise E. Sabatino *et.al.,* Recombinant canine B-domain–deleted FVIII exhibits high specific activity and is safe in the canine hemophilia A model, Blood, 12 November 2009, Vol. 114, No. 20, pp. 4562-4565).

The specific activity of OBI-1 and rpFVIIIv3 was determined by the methods as described in Example 5.

The results are depicted in Table 3 and Fig. 1. Due to its increased specific activities, lower amounts of prVIIIv3 per dose can be administered to a patient. It is expected that this decrease in dose will lead to a reduced immunogenicity, *i.e.* result in a lower probability that patients develop inhibitory antibodies.

Table 3: Specific activities of FVIII products as compared to rpVIIIv3

	Specific Activity in IU/mg Protein	Amt protein µg/dose^
Xynthia	7500	400
Kogenate FS	4000	750
Advate	4000	750
OBI-1	11000	273
rpVIIIv3	22000	136
Canine BDD* FVIII	34000	88

^Amt Protein = xxxx µg/dose. The numbers shown in the column for rpVIII3 mean that an amount of 136 µg per dose will be required. One dose is the required amount to raise the blood factor VIII level from 0% to 100%, i.e. the higher the specific activity (XXX units/mg), the less amount (in terms of µg) is required. Note that 1 mg = 1000µg. Given that a typical dose is 3000 units per patient, the amount of actual protein required = 3000/22000 = 0.136 mg = 136 µg.

*BDD stands for B-domain deleted and FL stands for full length

In conclusion, these results show that rpVIIIv3 has a significantly higher specific activity than the tested products that are presently commercialized, or under clinical development, for FVIII replacement therapy.

In particular, it is highly surprising a deletion of 27 amino acids, present in rpVIIIv3 but not in OBI-1, leads to a 50% increase in specific activity (as measured by the OSCA assay).

Example 5: Materials and methods

ONE-STAGE COAGULATION ASSAY - OSCA - METHOD

1. Dilute Reference Standard in Assay Buffer (10% Factor VIII deficient plasma + Owren's Veronal Buffer, per liter, 28 mmol of sodium barbital and 125 mmol of NaCl, pH 7.35) to target potency of 1.0 units/mL.
2. Dilute check standards, activity controls and samples in Assay Buffer to the target potency.
3. Load the calibration standard (reference) and reagents into the appropriate wells inside a Symex instrument. (Sysmex CA-1500, Coagulation Analyzer, Dade Behring # B4260-1500 (Siemans Corporation, Deerfield, IL)).
4. Load check standard, controls and samples into racks outside of the Sysmex instrument.
5. Assay sequence occurs automatically inside the instrument as follows:
    a. 5 uL of sample is diluted into 45 ul of assay buffer inside the reaction tube
    b. 50ul of factor VIII deficient plasma is added to the reaction tube
    c. 50ul of Dade Actin FS (purified soy phosphatides in 1.0 X 10^{-4}M Ellagic acid activator, Dade Behring, Liederbach, Germany) activated partial thromboplastin time reagent is added to the reaction tube and incubated for 60 seconds.

d. 50ul of Calcium Chloride (0.025M, Dade Behring, Liederbach, Germany) is added to the reaction tube and incubated for 240 seconds.

e. The clot time is measured up to a maximum time of 300 seconds.

6. The clot time is then correlated to the potency generated by the reference standard calibration curve.

SIZE-EXCLUSION (SEC) HPLC METHOD

An Agilent 1100 (Agilent Technologies, Santa Clara,CA) or a Waters Bioalliance HPLC system (Waters Corporation, Milford, MA) was and is typically used for the size exclusion method for determination of protein concentration. The typical size exclusion column used was a Superdex 200 from GE (Superdex 200 10.300 GL, 10 X 300mm, Cat # 17-5175-01 (GE Healthcare Lifesciences, Piscataway, NJ)). A general assay protocol is as follows:

1. A typical mobile phase was prepared that contained 400mM NaCl with 20mM TRIS, pH 7.4 and 0.01% polysorbate 80.
2. The mobile phase was run through the HPLC system and column until equilibration of the baseline was observed.
3. Standards were run to establish system suitability with a typical run time of 30 minutes for each sample.
4. Samples were typically loaded onto an auto sampler with a control chamber temperature of 4°C.
5. A calibration standard of known concentrations containing various amounts of standard samples, typically from 1, 3, 5, 10 and 25 µg of protein were loaded onto the column in volumes between 10 to 50 µl.
6. Samples of unknown concentrations were also loaded into the columns typically in volumes between 30 to 50 µl.
7. The HPLC system was programmed to run the samples automatically according the sequence set up.
8. The peak area of the calibration standards and the unknown samples were determined based on fluorescence detection with excitation and emission at 280nm and 340nm respectively.

9. A linear regression of the calibration standards was generated and the concentration of the unknown sample was determined against this calibration curve.

A typical example of the SEC profile of the calibration standards and the linear regression of the calibration curve is shown in Figure 3.

Example 6: Binding to von Willebrand Factor

Von Willebrand factor (vWF) is a multifunctional glycoprotein which circulates in plasma as a multimeric form complexed with Factor VIII (FVIII/vWF complex). The FVIII/vWF complex serves to protect the bound FVIII from early proteolysis in circulation *in vivo*.

Size exclusion chromatography (SEC) is used to characterize the binding in FIII/vWF complexes. A Superose 6 column (Superose 6 10/300GL, GE Healthcare #17-5172-01, flow rate = 0.5 mL/min) was selected for its known biocompatibility and high exclusion limit of 40 million Daltons (Mobile Phase formulation buffer: 2 mM CaCl2, 10 mM Tris pH 7.0, 300 mM NaCl, 0.01% PSB-80, 11 mM sucrose, 10 mM trisodium citrate). The differences in molecular weight between von Willebrand factor (dimeric @ ~ 500KD, Fitzgerald Cat# 30C-CP4003U, Lot # A09121050, Monomer Mol. Wt = ~260kD, Conc. = 77 µg/mL) and rpFVIII (~ 160 KD) is sufficient to resolve the two species.

The stoichiometry of binding was determined by titration of constant amount of vWF with increasing amounts of rpFVIII. The profile of the complexation formation between rpFVIII and vWF was determined from the SEC chromatogram and integration of the appropriate peaks. The soluble form of the resultant mixtures in the supernatant were used to determine the end point of the complexation from titration with increasing amounts of rpFVIII. The larger, stable complexes formed between rpFVIII and vWF migrate from the normal retention time to the exclusion limit of the column. When saturation occurs, increasing amounts of unbound rpFVIII will be observed in the SEC chromatogram. This method is used to distinguish differences and similarities in the properties of the variants.

Example 7: Thrombin Digestion Kinetics

SDS-PAGE

Recombinant porcine factor VIII (rpFVIII) molecules are heterodimers of approximately 160kD composed of a 78.5kD molecular weight light chain (A3-C1-C2, 765-1448) and a heavy chain ranging in molecular weight from 86.7kD (A1-A2). The

heavy chain of rpFVIII is heterogeneous and composed of three main variants which are formed upon secretion from the cell and may be cleaved by membrane bound proteases of the subtilisin family designated PACE (Paired Basic Amino Acid Cleavage Enzyme).

Like the human factor VIII, rpFVIII is transformed into an active form by limited proteolysis from thrombin. Thrombin activation of rpFVIII is specific for the cleavage site of the heavy chain at Arg(372)-Ser(373). The cleavage region for the Light Chain at Arg(805)-Ser(806) liberates a small 40 amino acid peptide fraction ranging from 765-804 (~4.4kD). The combination of these initial cleavages by thrombin form the activated recombinant porcine factor VIII as a heterotrimer of subunits designated as A1 (~50kD), A2 (~40kD), and A3-C1-C2 (~70kD). The cleaved rpFVIII A1 and A3-C1-C2 subunits retain the divalent metal ion-dependent linkage, whereas the A2 subunit is weakly associated with the A1–A3-C1-C2 dimer by primarily electrostatic interactions. In SDS-PAGE, these subunits are displayed as three distinct bands. The efficiency in conversion of the rpFVIII (2 peptide units) to three peptide units is characteristic of thrombin activation of the product. This method distinguishes the similarity and differences between the individual variants.

Two other orthogonal methods can be and were used to map the kinetics of thrombin digestion of FVIII. Denaturing reverse phase HPLC may be used; the peak profiles are expected to be similar to that of SDS-PAGE. Anion exchange chromatography, which retains the native form of the peptides, may also be used.

Reverse Phase HPLC

Equipment:

1. HPLC 1100 series: Agilent Technologies, Santa Clara, CA, USA; Model No. 61312A, Serial No. DE10907753.
2. Poroshell 5um 2.1X75mm column: Agilent Technologies, Santa Clara, CA, USA; Part No. 660750-909, Serial No. USVV001988.

Reagents:

1. Buffer A: 0.1% TFA (JT B Baker, Phillisburg, NJ; Cat No. 94700-00, Lot No. J23J00) in water
2. Buffer B: 0.1% TFA in Acetonitrile (JT Baker, Phillisburg, NJ; Cat No. 9017-03, Lot No. J38807).

Procedure:

1. Equilibrate the column for 60 minutes using 99%A and 1%B at 1ml/min.
2. Sample injections were 50µl in volume; 25 µl of reference standard was used as control.

3. An example of the method used to run the samples is shown Table 4.

Table 4:

Injection	Time (min)	Flow (ml/min)	%A	%B
1	0.00	2	99	1
2	2.00	2	99	1
3	3.00	2	64	36
4	3.50	2	64	36
5	4.00	2	61	39
6	5.00	2	61	39
7	5.10	2	58	42
8	6.50	2	58	42
9	6.51	2	56	44
10	7.20	2	56	44
11	7.21	2	40	60
12	8.50	2	40	60
13	8.51	2	10	90
14	9.00	2	10	90
15	9.10	2	99	1
16	10.00	2	99	1

Anion Exchange Chromatography

Equipment:

1. AllianceBio HPLC, Waters corporation, Milford, MA, USA; Model No. 2796; Serial No M08BA1199M.
2. Protein Pak Hi Res Q 5um 4.6X100mm column: Waters Corporation, Milford, MA, USA; Part No. 186004931, Serial No. 502N112561VE04.

Reagents:

Buffer A:

10mM Tris base (1.211g/L)

2mM Calcium Chloride (0.588g/L)

0.01% polysorbate 80 (1ml of 10% PS-80/L)

Filtered water up to 1L, pH 7.0

Buffer B:

10mM Tris base (1.211g/L)

2mM Calcium Chloride (0.588g/L)

0.01% polysorbate 80 (1ml of 10% PS-80/L)

1M Sodium Chloride (58.44g/L)

Filtered water upto 1L, pH 7.0

Procedure:

1. Equilibrate the column for 60min using 70%A and 30%B at 0.5ml/min
2. Sample injections were 50µl in volume; 10 µl of reference standard was used as control.
3. An example of the method used to run the samples is shown Table 5.

Table 5:

Injection	Time (min)	Flow (ml/min)	%A	%B
1	0.01	0.5	70	30
2	0.50	0.5	70	30
3	3.50	0.5	60	40
4	5.00	0.5	60	40
5	10.00	0.5	30	70
6	10.10	0.5	1	99
7	12.00	0.5	1	99
8	12.10	0.5	70	30
9	17.00	0.5	70	30

The skilled artisan would know and appreciate that these and other methods may be used to arrive at understanding recombinant Factor VIII proteins as described herein.

CLAIMS

1. An isolated, recombinant, fully or partially B-domain deleted factor VIII (FVIII) variant, the FVIII variant being devoid of an amino acid sequence corresponding to amino acids 716 to 742 of porcine factor VIII as depicted in Figure 2 or amino acids 714 to 740 of SEQ ID NO: 7.

2. The FVIII variant according to claim 1, comprising a sequence being at least 90% identical to SEQ ID NO: 1, the variant being devoid of the amino acid sequence corresponding to amino acids 716 to 742 of porcine factor VIII as depicted in Figure 2 or amino acids 714 to 740 of SEQ ID NO: 7.

3. The FVIII variant according to claim 2, wherein said variant shares at least 95, 96, 97, 98, 99, 99.5, 99.6, 99.7, 99.8 or 99.9% identity to SEQ ID NO:1.

4. The FVIII variant according to claim 3, wherein said variant shares 99.5, 99.6, 99.7, 99.8 or 99.9% identity to SEQ ID NO:1.

5. The FVIII variant according to claim 4, wherein said variant is SEQ ID NO:1.

6. An isolated, recombinant, fully or partially B-domain deleted factor VIII (FVIII) variant, the FVIII variant being devoid of an amino acid sequence corresponding to amino acids 716 to 742 of human factor VIII as depicted in Figure 2 or amino acids 714 to 740 of SEQ ID NO: 8.

7. The FVIII variant according to claim 6, comprising a sequence being at least 90% identical to SEQ ID NO: 1, the variant being devoid of the amino acid sequence corresponding to amino acids 716 to 742 of porcine factor VIII as depicted in Figure 2 or amino acids 714 to 740 of SEQ ID NO: 8.

8. The FVIII variant according to claim 7, wherein said variant shares at least 95, 96, 97, 98, 99, 99.5, 99.6, 99.7, 99.8 or 99.9% identity to SEQ ID NO:2.

9. The FVIII variant according to claim 8, wherein said variant shares 99.5, 99.6, 99.7, 99.8 or 99.9% identity to SEQ ID NO:2.

10. The FVIII variant according to claim 9, wherein said variant is SEQ ID NO:2.

11. The FVIII variant according to any one of the preceding claims, wherein the FVIII variant is partially B-domain deleted and the remaining portion of the B-domain is selected from a sequence consisting of SEQ ID NO: 4 or SEQ ID NO:5.

12. A polynucleotide encoding a polypeptide according any one of the preceding claims.

13. An expression vector comprising a polynucleotide according to claim 12.

14. A mammalian cell comprising an expression vector according to claim 13.

15. A method for producing a FVIII variant according to any one of claims 1 to 10, comprising the steps of :

a. Culturing a mammalian cell according to claim 14; and

b. Isolating from the mammalian cell the FVIII variant.

16. The method according to claim 15, further comprising the step of

c. Formulating the factor VIII variant together with appropriate excipients into a pharmaceutical composition.

17. A pharmaceutical composition comprising a factor VIII variant according to any one of claims 1 to 10.

18. A method of treating a patient suffering from hemophilia comprising administering a therapeutically effective amount of FVIII variant according to any one of claims 1 to 10 to a patient in need thereof, thereby treating the hemophilia in said patient.

19. A FVIII variant according to any one of claims 1 to 10 for use in treating hemophilia.

20. The method or use according to claim 18 or 19, wherein said hemophilia is hemophilia A.

21. The method or use according to claim 18 or 19, wherein said hemophilia is acquired hemophilia.

22. The method or use according to any one of claims 18 to 21, wherein said hemophilia is treated in patients having developed human FVIII antibodies.

23. The method or use according to any one of claims 18 to 22, wherein the factor VIII is administered in an amount of no more than 200 µg/dose or 150 µg/dose or 140 µg/dose.