



US 20160102133A1

(19) **United States**(12) **Patent Application Publication**
XIAO et al.(10) **Pub. No.: US 2016/0102133 A1**(43) **Pub. Date: Apr. 14, 2016**(54) **MUTANT FACTOR VIII COMPOSITIONS AND METHODS****Publication Classification**(71) Applicant: **Weidong XIAO**, Fort Washington, PA (US)(51) **Int. Cl.**
C07K 14/755 (2006.01)(72) Inventors: **Weidong XIAO**, Fort Washington, PA (US); **Wenjing CAO**, Ardmore, PA (US); **Biao DONG**, Philadelphia, PA (US)(52) **U.S. Cl.**
CPC **C07K 14/755** (2013.01); **A61K 38/00** (2013.01)(21) Appl. No.: **14/893,878**(57) **ABSTRACT**(22) PCT Filed: **Jun. 24, 2014**(86) PCT No.: **PCT/US2014/043777**

§ 371 (c)(1),

(2) Date: **Nov. 24, 2015****Related U.S. Application Data**

(60) Provisional application No. 61/838,867, filed on Jun. 24, 2013.

In one aspect, present invention provides a recombinant mutant human factor VIII having increased expression and/or secretion as compared to wild-type factor VIII. In certain embodiments, the recombinant factor VIII includes one or more amino acid substitution(s) selected from the group consisting of I86, Y105, A108, D115, Q117, F129, G132, H134, M147 and L152. In other aspects, the present invention provides FVIII encoding nucleic acids, FVIII-expression vectors, as well as methods of using the modified FVIII genes in the treatment of FVIII deficiencies, such as hemophilia A.

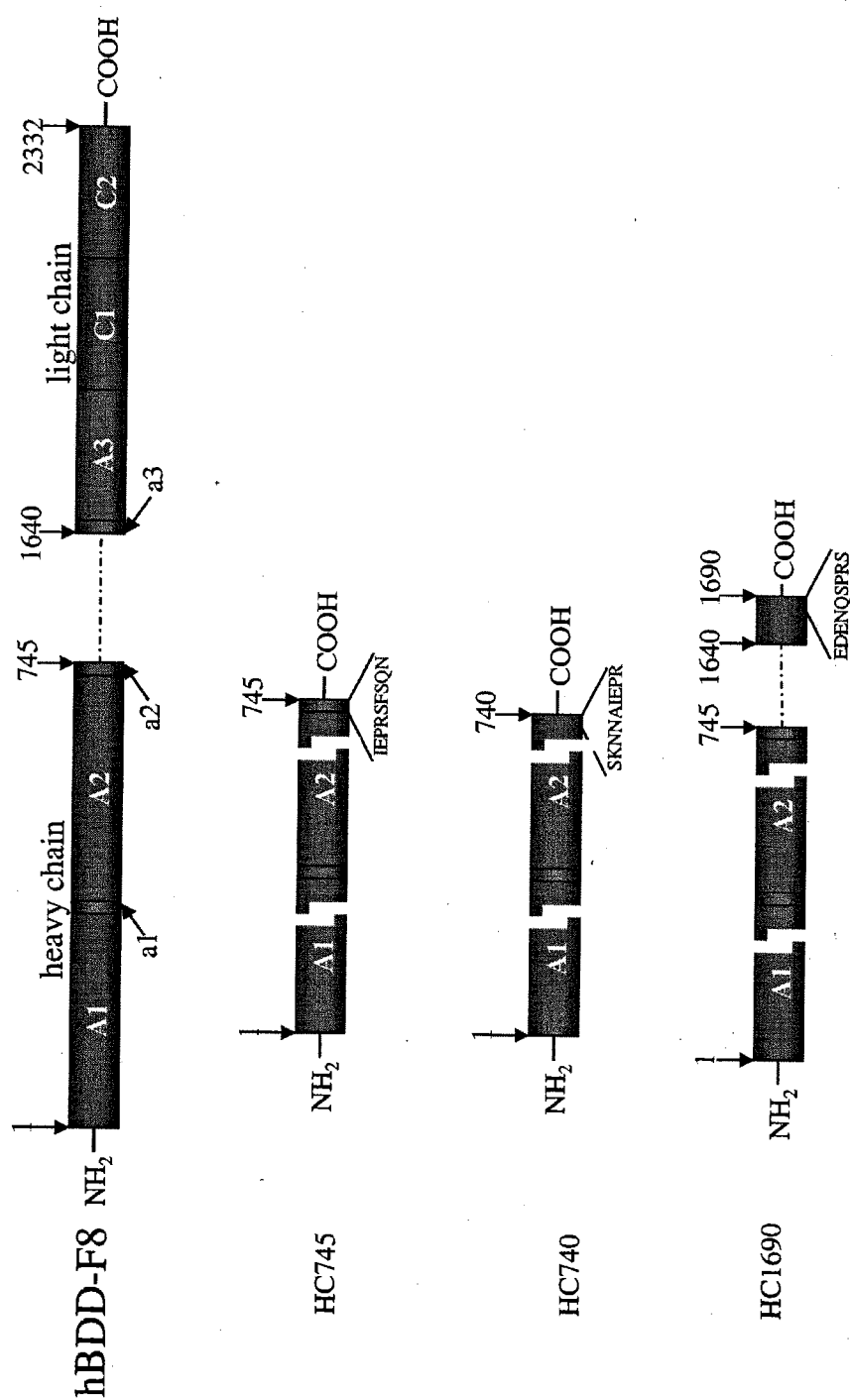


FIG. 1

[illegible]

FIG. 2

	1			50
hF8	ATRRYYLGAV	ELSWDYMQSD	LGELPVDARF	PPRVPKSFPP
hF8-x5	ATRRYYLGAV	ELSWDYMQSD	LGELPVDARF	PPRVPKSFPP
	51			100
hF8	FVEFTDHLFN	IAKPRPPWMG	LLGPTIQAEV	YDTVVITLKN
hF8-x5	FVEFTDHLFN	IAKPRPPWMG	LLGPTIQAEV	YDTVVITLKN
	101			150
hF8	VGVSYYWKASE	GAEYDDQTSQ	REKEDDKVFP	GGSHYYVWQV
hF8-x5	VGVSYYWKSSE	GAEYDDQTSQ	REKEDDKVFP	GGSHYYVWQV
	151			200
hF8	PLCLTYSYLS	HVDLVKDLNS	GLIGALLVCR	EGSLAKEKTQ
hF8-x5	PPCLTYSYLS	HVDLVKDLNS	GLIGALLVCR	EGSLAKEKTQ

FIG. 3

Human factor VIII amino acids	Exemplary substitutions with enhanced secretion
I86	V,L,M
Y105	F,W
A108	S,G,T,P
D115	E,N,H,Q,R,K
Q117	H,N,E,D,R,K
F129	L,V,I,M,P,T,K
G132	K,E,D,R,T,M,N,S,W
H134	Q,G,Y,N,E,D,R,K
M147	T,A,G,S,P
L152	P,S,G,T

FIG. 4

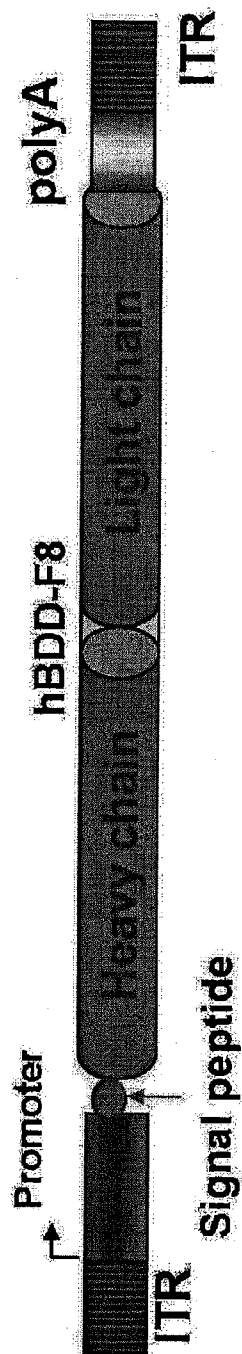


FIG. 5

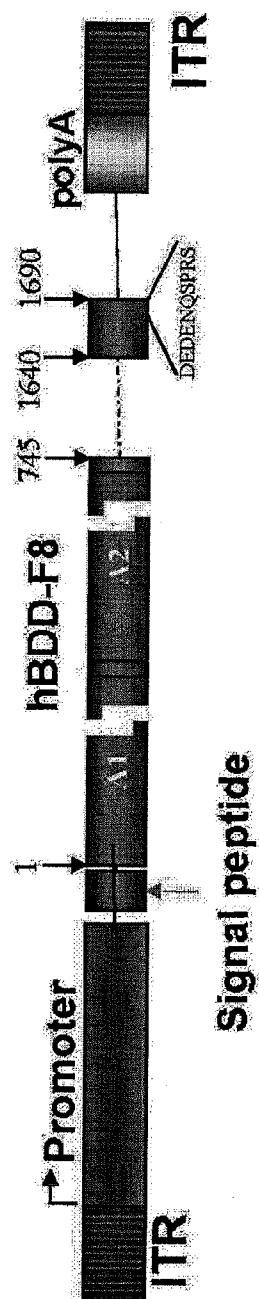


FIG. 6

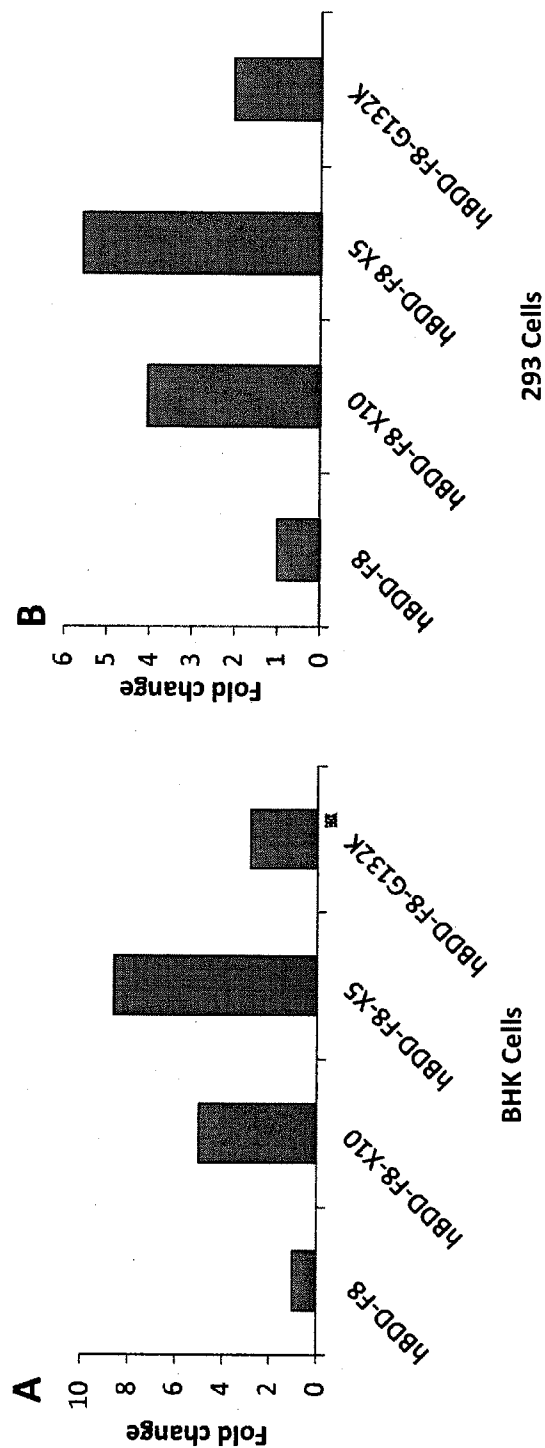


FIG. 7

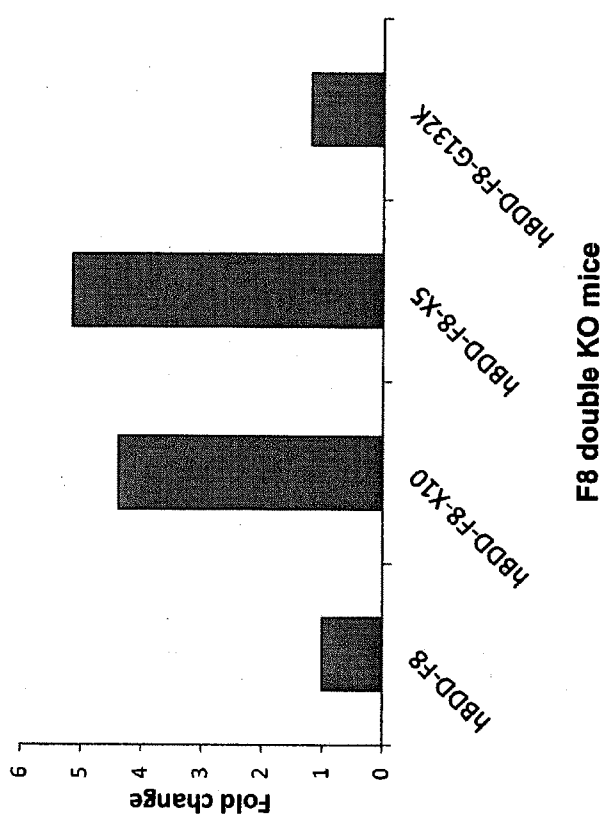


FIG. 8

Substitutions of amino acids in hBDD-F8-X10	% secretion/activity relative to hBDD-F8-X10
hBDD-F8-X10-V86I	80
hBDD-F8-X10-S108A	71
hBDD-F8-X10-E115D	89
hBDD-F8-X10-H117Q	88
hBDD-F8-X10-L129F	77
hBDD-F8-X10-K132G	55
hBDD-F8-X10-T147M	75
hBDD-F8-X10-P152L	63
hBDD-F8-X10-6p	42
hBDD-F8-X10	100

FIG. 9

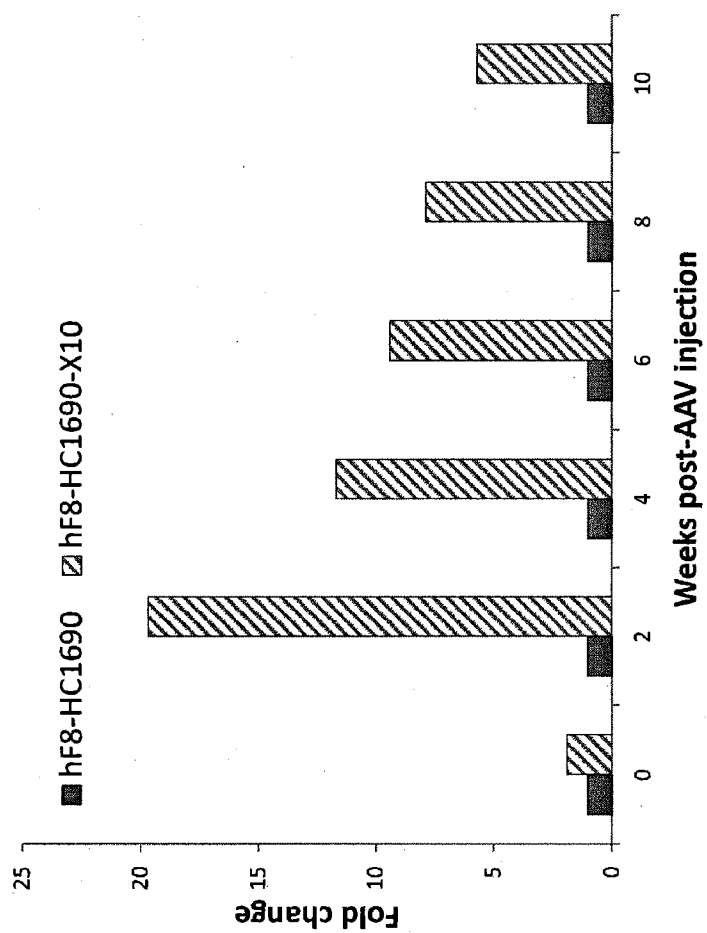


FIG. 10

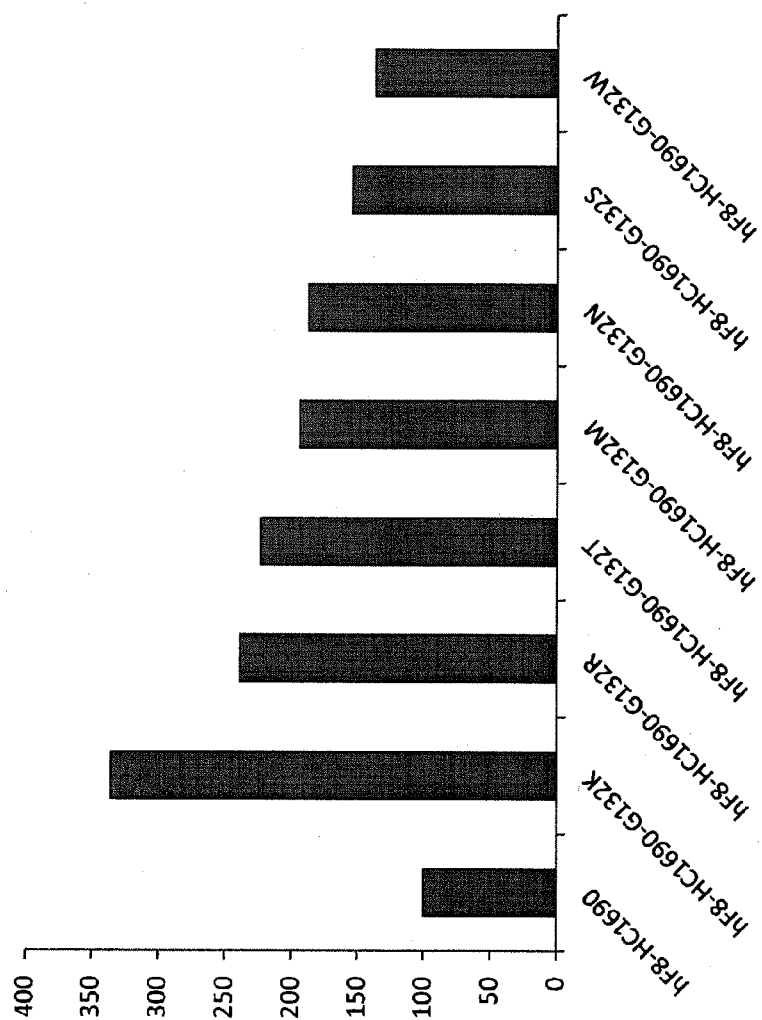


FIG. 11

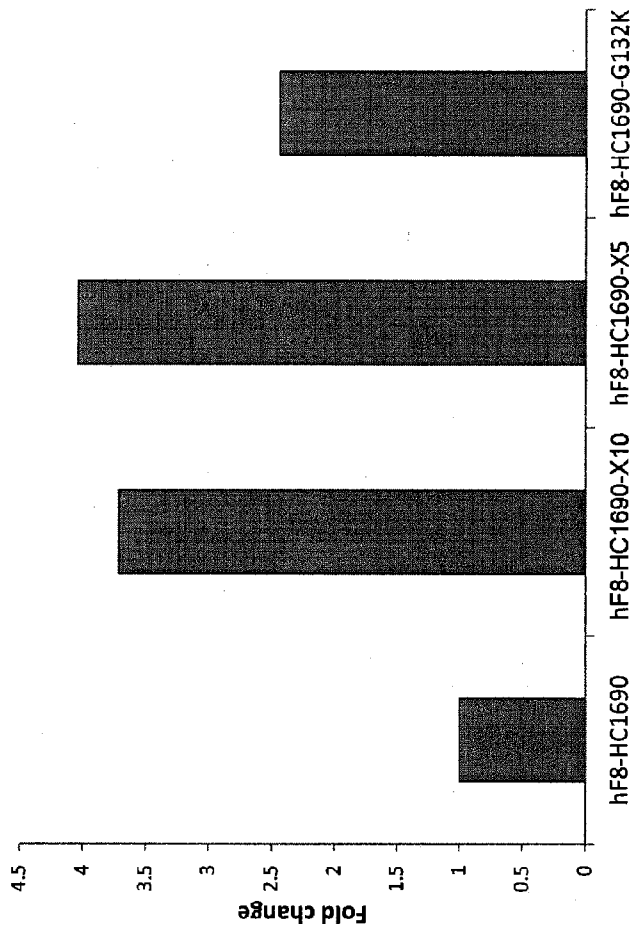


FIG. 12

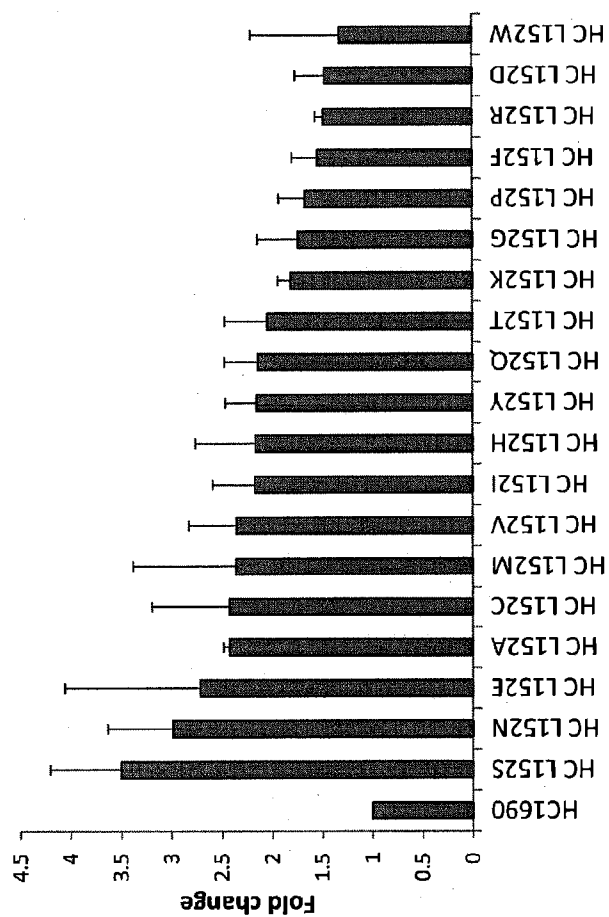


FIG. 13

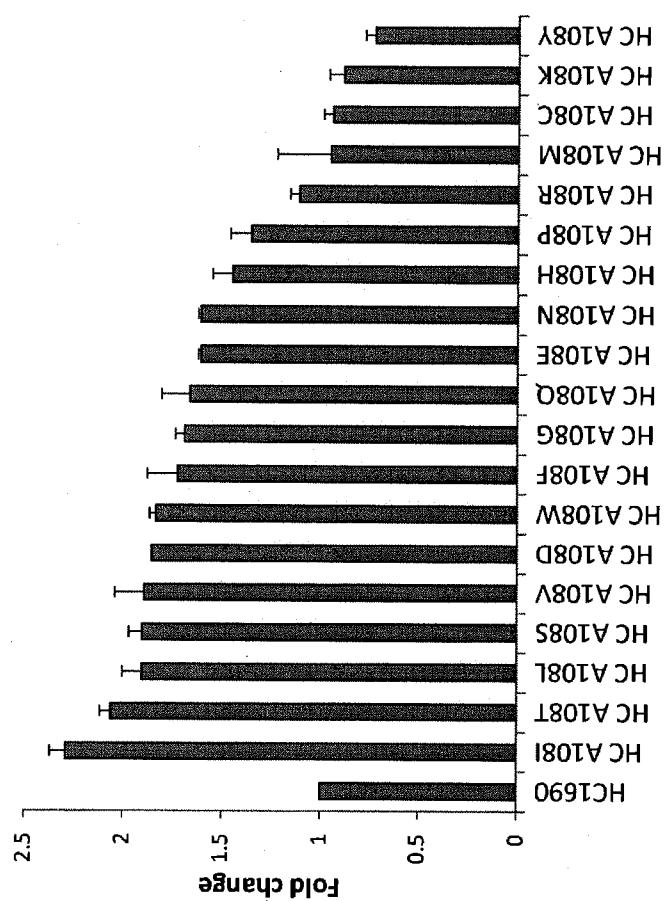


FIG. 14

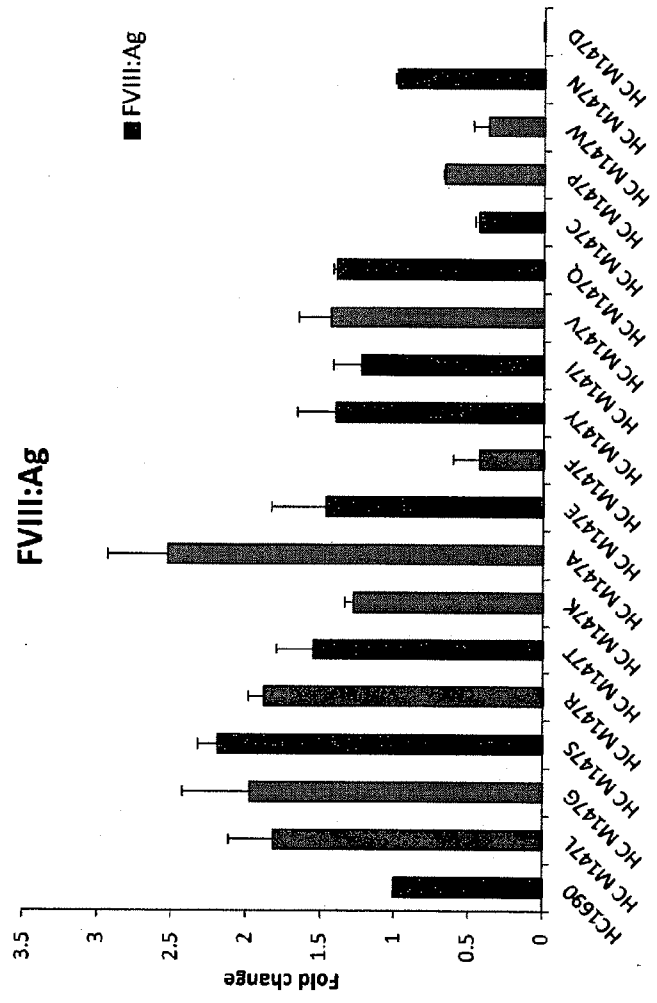


FIG. 15

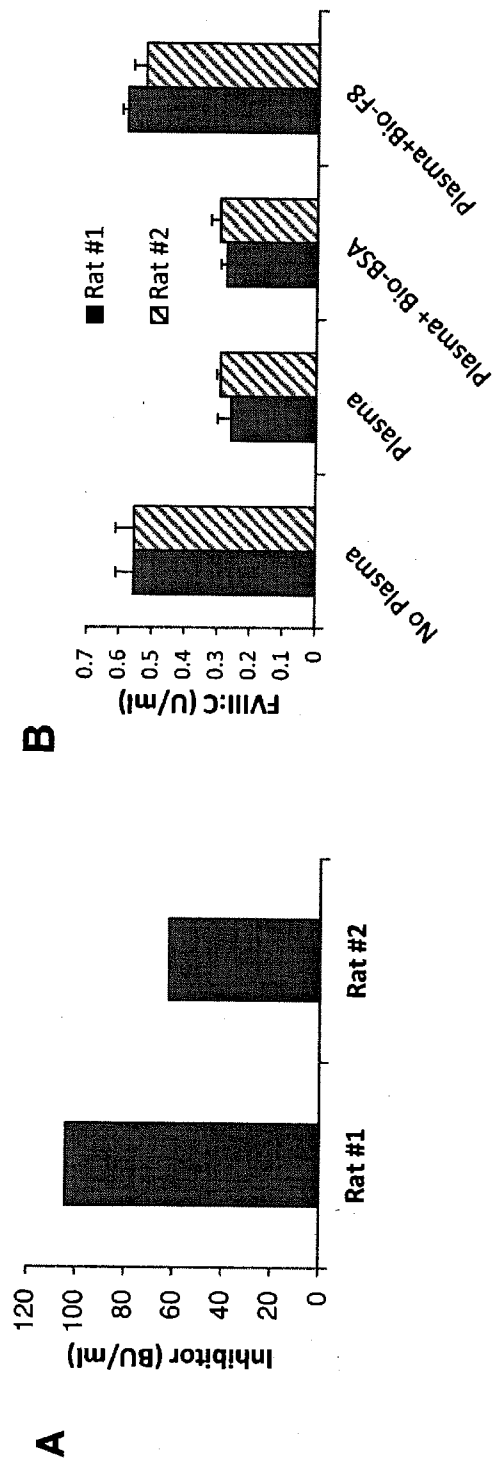


FIG. 16

MUTANT FACTOR VIII COMPOSITIONS AND METHODS

[0001] This application claims priority to U.S. Provisional Patent Application Ser. No. 61/838,867, filed on Jun. 24, 2013. The entirety of the aforementioned application is incorporated herein by reference.

GOVERNMENT LICENSE RIGHTS

[0002] This invention was made with U.S. Government support under grant HL084381 from the National Institutes of Health. The U.S. Government thus may have certain license rights in this invention.

FIELD

[0003] The present invention relates to recombinant human factor VIII mutants exhibiting higher expression levels than the corresponding wild-type human factor VIII. The present invention also relates to methods of making and using the recombinant human factor VIII mutants.

BACKGROUND

[0004] Hemophilia A, the most common of the severe, inherited bleeding disorders, results from a deficiency or defect in the plasma protein factor VIII. In patients with Hemophilia A, the blood does not clot properly resulting in excessive bleeding when the hemophiliac is injured. Treatment consists of replacement therapy using preparations of (purified) plasma or the recombinant protein.

[0005] 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 protein precursor is converted to a protease that cleaves the next protein precursor in the series. Cofactors are required at most of the steps.

[0006] Factor VIII circulates as an inactive, non-covalent, metal ion-dependent heterodimer precursor in blood, bound tightly and non-covalently to von Willebrand factor. This procofactor form of the protein contains a heavy chain (HC) comprised of A1(a1)A2(a2)B domains and a light chain (LC) comprised of (a3)A3C1C2 domains, with the lower case a representing short (~30-40 residue) segments rich in acidic residues. Factor VIII is proteolytically activated by proteolytic cleavages at the A1A2, A2B and A3A3 junctions catalyzed by thrombin or factor Xa, which serves to dissociate it from von Willebrand factor and activate its procoagulant function in the cascade. In its active form, the protein factor VIIIa is a cofactor that increases the catalytic efficiency of the serine protease factor IXa in the membrane-dependent conversion of zymogen factor X to the serine protease, factor Xa factor by several orders of magnitude.

[0007] Gene therapy has been proposed as treatment modality for supplementing deficiencies in clotting factors in hemophiliacs and there have been attempts to engineer FVIII constructs that are suitable for treatment of humans. For example, Connelly, et al. reported that treatment of FVIII-deficient mice with human FVIII-encoding adenoviral vectors resulted in expression of biologically active human FVIII (Connelly, et al., Blood, Vol. 91, No. 9 (1998), pp. 3273-3281). Sarkar, et al. reported that use of AAV8 serotype in

combination with FVIII corrected plasma FVIII activity in mouse models (Sarkar, et al., Blood, Vol. 103, No. 4 (2004), pp. 1253-1260).

[0008] However, as in many areas of gene therapy, theory is much more straightforward than successful, effective implementation. Difficulties in implementation of gene therapy techniques include problems encountered in the use of viruses as gene vectors and insufficient expression levels of FVIII. For example, human FVIII secretes very inefficiently and the yield is logs lower comparing to similar proteins such as factor V. Further, while viruses are effective as gene vectors because they can be used to transduce cells leading to protein expression in vivo, the proteins coating the virus particle may activate the body's immune system.

[0009] Thus, in view of the foregoing, there is a need for approaches that can efficiently express the target FVIII protein in sufficient quantity to reduce the required dose of viral vector to tolerable levels.

SUMMARY

[0010] The present invention provides modified factor VIII (FVIII) proteins, FVIII encoding nucleic acids, and FVIII-expression vectors, as well as methods of using the modified FVIII genes in the treatment of FVIII deficiencies, such as hemophilia A.

[0011] In one aspect, present invention provides a mutant human factor VIII having increased expression or secretion as compared to wild-type factor VIII.

[0012] In one embodiment, the recombinant mutant human factor VIII includes one or more amino acid substitution(s) selected from the group consisting of I86, Y105, A108, D115, Q117, F129, G132, H134, M147, L152 and combinations thereof.

[0013] In another embodiment, the recombinant mutant human factor VIII includes one or more amino acid substitution(s) selected from the group consisting of I86V, Y105F, A108S, D115E, Q117H, F129L, G132K, H134Q, M147T, L152P and combinations thereof.

[0014] In another embodiment, the recombinant mutant human factor VIII includes amino acid substitutions in each of the amino acids I86, Y105, A108, D115, Q117, F129, G132, H134, M147 and L152.

[0015] In another embodiment, the recombinant mutant human factor VIII includes the amino acid substitutions I86V, Y105F, A108S, D115E, Q117H, F129L, G132K, H134Q, M147T and L152P.

[0016] In another embodiment, the recombinant mutant human factor VIII includes one or more amino acid substitution(s) selected from the group consisting of I86, A108, G132, M147, L152 and combinations thereof.

[0017] In another embodiment, the recombinant mutant human factor VIII includes one or more amino acid substitution(s) selected from the group consisting of I86V, A108S, G132K, M147T, L152P and combinations thereof.

[0018] In another embodiment, the recombinant mutant human factor VIII includes amino acid substitutions in each of I86V, A108S, G132K, M147T and L152P.

[0019] In another embodiment, the recombinant mutant human factor VIII includes the amino acid substitutions I86V, A108S, G132K, M147T and L152P.

[0020] In other embodiments, the human factor VIII mutants further include a deletion in the B domain of human factor VIII.

[0021] In other embodiments, the human factor VIII mutants further include the $\alpha 2$ and/or $\alpha 3$ domain(s) of human factor VIII.

[0022] In another aspect, the present invention provides isolated polynucleotide sequences encoding the human factor VIII mutants described herein.

[0023] In yet another aspect, the present invention provides an expression vector operatively linked to the polynucleotides encoding the human factor VIII mutants described herein.

[0024] In a further aspect, the present invention provides a pharmaceutical composition comprising an expression vector operatively linked to the polynucleotides encoding the human factor VIII mutants described herein.

[0025] In another aspect, the present invention provides a method for treating a patient with a factor VIII deficiency comprising administering to a patient in need thereof a pharmaceutical composition comprising an expression vector operatively linked to the polynucleotides encoding a human factor VIII mutant described herein in an amount effective for treating the factor VIII deficiency.

[0026] In yet another aspect, the present invention provides a method for expressing a human factor VIII polypeptide mutant comprising: (a) transforming a host cell with an expression vector operatively linked to a polynucleotides encoding a human factor VIII mutant according to the present invention; (b) growing the host cell under conditions suitable for expressing the human factor VIII polypeptide mutant; and (c) purifying the human factor VIII polypeptide mutant from host cells expressing said mutant.

BRIEF DESCRIPTION OF DRAWINGS

[0027] FIG. 1 illustrates the structural domains of the human factor VIII heavy and light chains, including several heavy chain constructions utilized in the present invention.

[0028] FIG. 2 is a graph showing an alignment of the first 200 amino acids of the secreted human factor VIII heavy chain alongside a modified factor VIII with 10 exemplary substitutions for enhanced secretion.

[0029] FIG. 3 is a graph showing the alignment of the first 200 amino acids of the secreted human factor VIII heavy chain alongside a modified human factor VIII mutant with 5 exemplary substitutions for enhanced secretion.

[0030] FIG. 4 summarizes exemplary amino acids determined to affect human factor VIII secretion.

[0031] FIGS. 5 and 6 depict exemplary AAV vectors for expressing B-domain deleted human factor VIII mutants or human factor VIII heavy chain.

[0032] FIG. 7 shows a comparison of the secretion of different human factor VIII mutants in BHK cells (panel A) or 293 cells (panel B).

[0033] FIG. 8 shows a comparison of the secretion of different human factor VIII mutants in secretion in vivo. Plasmids pAAV-CB-hBDDF8 (carrying human factor VIII with B-domain deleted), pAAV-CB-hBDDF8-X10 (carrying hF8-BDD with 10 substitutions), pAAV-CB-hBDD-F8-X5 (carrying hBDDF8 with 5 substitutions) or pAAV-CB-hBDD-F8-G312K (hF8 with G132K substitutions) were injected in factor VIII double knock-out Blab/c mice.

[0034] FIG. 9 show a comparison of the secretion of different human factor VIII mutants in 293 cells. Amino acids in hBDD-F8-X10 were reverted back to their corresponding wild type amino acids. In hBDD-F8-X10-6p, 6 of 10 amino

acids in hBDD-F8-X10 differing from wild type F8 were reverted back to their wild type amino acids.

[0035] FIG. 10 shows a comparison of the expression and secretion of a mutant factor VIII (F8) heavy chain (HC1690-X10) in an AAV vector construct with a wild type hF8-HC1690 AAV vector construct (AAV/hF8HC1690) in vivo.

[0036] FIG. 11 shows a comparison of the secretion of different G132 factor VIII heavy chain mutants in 293 cells.

[0037] FIG. 12 shows a comparison of the secretion of different factor VIII heavy chain mutants in expression/secretion in factor VIII double knock-out Blab/c mice.

[0038] FIG. 13 shows a comparison of the secretion of different L152 factor VIII heavy chain mutants in 293 cells.

[0039] FIG. 14 shows a comparison of the secretion of different A108 factor VIII heavy chain mutants in 293 cells.

[0040] FIG. 15 shows a comparison of the secretion of different M147 factor VIII heavy chain mutants in 293 cells.

[0041] FIG. 16 shows that there was no formation of neutralizing antibodies against 5 mutated amino acids in the F8-/- rat model.

DETAILED DESCRIPTION

[0042] Various terms relating to the biological molecules of the present invention are used hereinabove and also throughout the specification and claims.

[0043] The phrase “secretion enhanced factor VIII (seFVIII, seF8)” refers to a modified FVIII (F8) which has been genetically altered such that the encoded protein exhibits at least 10% or 20% or 50% or 100% increase in secretion when compared to unmodified FVIII. The nucleotide sequences described herein are readily obtainable from GenBank. For human FVIII, see Accession No. NG-011403.1.

[0044] The phrase “BDD” refers to a “B-domainless” factor VIII (F8 or FVIII) mutant lacking the B domain.

[0045] The phrase “one or more” followed by a list of elements or species is intended to encompass any permutation of elements or species in the list. Thus, for example, the phrase “one or more substitution mutations selected from the group consisting of A, B, C, D, E and F” may include any combination of substitution mutations containing A, B, C, D, E and/or F.

[0046] As used herein, ranges may be expressed from one particular integer value to another particular integer value. When such a range is expressed, it should understand that any and all integer values within that range define separate embodiments according to the present invention and that the full scope of embodiments includes within the range further includes any and all sub-ranges between any pair of integer values in the initial range.

[0047] With reference to nucleic acids of the invention, the term “isolated nucleic acid”, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous (in the 5' and 3' directions) in the naturally occurring genome of the organism from which it originates. For example, the “isolated nucleic acid” may comprise a DNA or cDNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the DNA of a prokaryote or eukaryote. The nucleic acid codons can be optimized for enhanced expression in the mammalian cells.

[0048] With respect to RNA molecules of the invention, the term “isolated nucleic acid” primarily refers to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from RNA molecules

with which it would be associated in its natural state (i.e., in cells or tissues), such that it exists in a “substantially pure” form (the term “substantially pure” is defined below).

[0049] With respect to protein, the term “isolated protein” or “isolated and purified protein” is sometimes used herein. This term refers primarily to a protein produced by expression of an isolated nucleic acid molecule of the invention. Alternatively, this term may refer to a protein which has been sufficiently separated from other proteins with which it would naturally be associated, so as to exist in “substantially pure” form.

[0050] The term “promoter region” refers to the transcriptional regulatory regions of a gene, which may be found at the 5' or 3' side of the coding region, or within the coding region, or within introns.

[0051] The term “vector” refers to a small carrier DNA molecule into which a DNA sequence can be inserted for introduction into a host cell where it will be replicated. An “expression vector” is a specialized vector that contains a gene or nucleic acid sequence with the necessary regulatory regions needed for expression in a host cell.

[0052] The term “operably linked” means that the regulatory sequences necessary for expression of a coding sequence are placed in the DNA molecule in the appropriate positions relative to the coding sequence so as to effect expression of the coding sequence. This same definition is sometimes applied to the arrangement of coding sequences and transcription control elements (e.g., promoters, enhancers, and termination elements) in an expression vector. This definition is also sometimes applied to the arrangement of nucleic acid sequences of a first and a second nucleic acid molecule wherein a hybrid nucleic acid molecule is generated.

[0053] The term “substantially pure” refers to a preparation comprising at least 50-60% by weight the compound of interest (e.g., nucleic acid, oligonucleotide, protein, etc.). More preferably, the preparation comprises at least 75% by weight, and most preferably 90-99% by weight, of the compound of interest. Purity is measured by methods appropriate for the compound of interest (e.g., chromatographic methods, agarose or polyacrylamide gel electrophoresis, HPLC analysis, and the like).

[0054] The phrase “consisting essentially of” when referring to a particular nucleotide sequence or amino acid sequence means a sequence having the properties of a given SEQ ID NO. For example, when used in reference to an amino acid sequence, the phrase includes the sequence per se and molecular modifications that would not affect the basic and novel characteristics of the sequence.

[0055] The term “oligonucleotide,” as used herein refers to primers and probes of the present invention, and is defined as a nucleic acid molecule comprised of two or more ribo- or deoxyribonucleotides, preferably more than three. The exact size of the oligonucleotide will depend on various factors and on the particular application for which the oligonucleotide is used. The term “probe” as used herein refers to an oligonucleotide, polynucleotide or nucleic acid, either RNA or DNA, whether occurring naturally as in a purified restriction enzyme digest or produced synthetically, which is capable of annealing with or specifically hybridizing to a nucleic acid with sequences complementary to the probe. A probe may be either single-stranded or double-stranded. The exact length of the probe will depend upon many factors, including temperature, source of probe and method of use. For example, for diagnostic applications, depending on the complexity of the

target sequence, the oligonucleotide probe typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides.

[0056] The term “percent identical” is used herein with reference to comparisons among nucleic acid or amino acid sequences. Nucleic acid and amino acid sequences are often compared using computer programs that align sequences of nucleic or amino acids thus defining the differences between the two. Comparisons of nucleic acid sequences may be performed using the GCG Wisconsin Package version 9.1, available from the Genetics Computer Group in Madison, Wis. For convenience, the default parameters (gap creation penalty=12, gap extension penalty=4) specified by that program are intended for use herein to compare sequence identity. Alternately, the Blastn 2.0 program provided by the National Center for Biotechnology Information (found on the world wide web at ncbi.nlm.nih.gov/blast/; Altschul, et al., 1990, J Mol Biol 215:403-410) using a gapped alignment with default parameters, may be used to determine the level of identity and similarity between nucleic acid sequences and amino acid sequences.

[0057] A “corresponding” nucleic acid or amino acid or sequence of either, as used herein, is one present at a site in a factor VIII or hybrid factor VIII molecule or fragment thereof that has the same structure and/or function as a site in the factor VIII molecule of another species, although the nucleic acid or amino acid number may not be identical. A sequence “corresponding to” another factor VIII sequence substantially corresponds to such sequence, and hybridizes to the human factor VIII DNA sequence designated SEQ ID NO:1 under stringent conditions. A sequence “corresponding to” another factor VIII sequence also includes a sequence that results in the expression of a factor VIII or claimed procoagulant hybrid factor VIII or fragment thereof and would hybridize to a nucleic molecule comprising SEQ ID NO:1 but for the redundancy of the genetic code.

[0058] A “unique” amino acid residue or sequence, as used herein, refers to an amino acid sequence or residue in the factor VIII molecule of one species that is different from the homologous residue or sequence in the factor VIII molecule of another species.

[0059] “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 protein in a standard assay in which the clotting time of human factor VIII deficient plasma is compared to that of normal human plasma. 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. Hybrid human/porcine factor VIII has coagulation activity in a human factor VIII assay. This activity, as well as that of other hybrid or hybrid equivalent factor VIII molecules or fragments thereof, may be less than, equal to, or greater than that of either plasma-derived or recombinant human factor VIII.

[0060] “Subunits” of human or animal factor VIII, as used herein, are the heavy and light chains of the protein. The heavy chain of factor VIII contains three domains, A1, A2, and B. The light chain of factor VIII also contains three domains, A3, C1, and C2.

[0061] The terms “epitope”, “antigenic site”, and “antigenic determinant”, as used herein, are used synonymously and are defined as a portion of the human, animal, hybrid, or

hybrid equivalent factor VIII or fragment thereof that is specifically recognized by an antibody. It can consist of any number of amino acid residues, and it can be dependent upon the primary, secondary, or tertiary structure of the protein. In accordance with this disclosure, a hybrid factor VIII, hybrid factor VIII equivalent, or fragment of either that includes at least one epitope may be used as a reagent in the diagnostic assays described below. In some embodiments, the hybrid or hybrid equivalent factor VIII or fragment thereof is not cross-reactive or is less cross-reactive with all naturally occurring inhibitory factor VIII antibodies than human or porcine factor VIII.

[0062] The term “immunogenic site”, as used herein, is defined as a region of the human or animal factor VIII, hybrid or hybrid equivalent factor VIII, or fragment thereof that specifically elicits the production of antibody to the factor VIII, hybrid, hybrid equivalent, or fragment in a human or animal, as measured by routine protocols, such as immunoassay, e.g., ELISA, or the Bethesda assay, described herein. It can consist of any number of amino acid residues, and it can be dependent upon the primary, secondary, or tertiary structure of the protein. In some embodiments, the hybrid or hybrid equivalent factor VIII or fragment thereof is nonimmunogenic or less immunogenic in an animal or human than human or porcine factor VIII.

[0063] “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 protein it encodes.

[0064] In one aspect, the present invention relates to a recombinant factor VIII mutant molecule (e.g., protein or nucleic acid) characterized by increased expression and/or secretion as compared to wild-type factor VIII.

[0065] Exemplary human factor VIII cDNA (nucleotide) and predicted amino acid sequences are shown in SEQ ID NOs: 1 and 2, respectively. Human factor VIII is synthesized and secreted as an approximately 300 kDa single chain protein of 2332 amino acids with internal sequence homologies defining a series of structural “domains” as follows: NH₂-A1-a1-A2-a2-B-a3-A3-C1-C2-COOH (FIG. 4). As used herein, a factor VIII “domain” is defined by a continuous sequence of amino acids characterized by e.g., internal amino acid sequence identity to structurally related domains and by sites of proteolytic cleavage by thrombin. Further, the terms “domainless” or “lacking a domain” should be understood to mean that at least 95% or 100% of the domain has been deleted. Unless otherwise specified, factor VIII domains are defined by the following amino acid residues in the human factor VIII amino acid sequence set forth in SEQ ID NO:2:

[0066] A1, residues A1a1-Arg372

[0067] A2, residues Ser373-Arg740

[0068] B, residues Ser741-Arg1648;

[0069] a3, residues P1640-Arg1649;

[0070] A3, residues Ser1690-Ile2032;

[0071] C1, residues Arg2033-Asn2172; and

[0072] C2, residues Ser2173-Tyr2332

[0073] The A3-C1-C2 sequence includes residues Ser1690-Tyr2332. The remaining sequence, residues Glu1649-Arg1689, is usually referred to as the factor VIII light chain activation peptide (FIG. 1). Factor VIII is proteolytically activated by thrombin or factor Xa, which dis-

sociates it from von Willebrand factor, forming factor VIIIa, which has procoagulant function. The biological function of factor VIIIa is to increase the catalytic efficiency of factor IXa toward factor X activation by several orders of magnitude. Thrombin-activated factor VIIIa is a 160 kDa A1/A2/A3-C1-C2 heterotrimer that forms a complex with factor IXa and factor X on the surface of platelets or monocytes.

[0074] A cDNA sequence encoding the wild-type human factor VIII has the nucleotide sequence set forth in SEQ ID NO:1. In SEQ ID NO:1, the first 57 nucleotides of the factor VIII open reading frame encodes a signal peptide sequence which is typically cleaved off from the mature factor VIII protein represented by SEQ ID NO:2.

[0075] Preferred recombinant factor VIII mutants include or encode one or more amino acid substitutions in the region from aa 86 to aa 152 of the wild-type human factor VIII amino acid sequence set forth in SEQ ID NO:2. Substitutions within any of these positions may employ any of the other 19 amino acids.

[0076] With reference to mutants described herein, the notion represented by “(amino acid a)-(SEQ ID NO:2 amino acid #b)-(amino acid c)” should be understood to mean that wild type amino acid a (one-letter code) at amino acid number b of SEQ ID NO:2 has been mutated to amino acid c.

[0077] In certain preferred embodiments, the human factor VIII polypeptide mutant comprises amino acid substitution (s) in one or more amino acids in SEQ ID NO:2 selected from the group consisting of I86, Y105, A108, D115, Q117, F129, G132, H134, M147 and L152. Further, the human factor VIII mutants may include any permutation of mutations encompassing these ten amino acid sites. Exemplary human factor VIII mutants are described in FIG. 4.

[0078] An exemplary recombinant factor VIII of this invention includes a point mutation involving a substitution at I86 of SEQ ID NO:2. A preferred substitution includes valine (i.e., I86V). Further preferred substitutions include leucine (I86L) and methionine (I86M).

[0079] Another exemplary recombinant factor VIII includes a point mutation involving a substitution at Y105 of SEQ ID NO:2. The substitution may include any of the other 19 amino acids. Preferred substitutions include Y105F and Y105W.

[0080] Another exemplary recombinant factor VIII includes a point mutation involving a substitution of at positions A108 of SEQ ID NO:2. A preferred substitution includes: A108S. Further preferred substitutions include A108S, A108G, A108T and A108P.

[0081] Another exemplary recombinant factor VIII includes a point mutation involving a substitution of at positions D115 of SEQ ID NO:2. A preferred substitution includes: D115E. Further preferred substitutions include D115N, D115H, D115Q, D115R and D115K.

[0082] Another exemplary recombinant factor VIII includes a point mutation involving a substitution of at positions Q117 of SEQ ID NO:2. A preferred substitution includes: Q117H. Further preferred substitutions include Q117N, Q117E, Q117D, Q117R and Q117K.

[0083] Another exemplary recombinant factor VIII includes a point mutation involving a substitution of at positions F129 of SEQ ID NO:2. A preferred substitution includes: F129L. Further preferred substitutions include F129V, F129I, F129M, F129P, F129T and F129K.

[0084] Another exemplary recombinant factor VIII includes a point mutation involving a substitution of at posi-

tions G132 of SEQ ID NO:2. A preferred substitution include: G132K. Further preferred substitutions include G132E, G132D, G132R, G132T, G132M, G132N, G132S and G132W.

[0085] Another exemplary recombinant factor VIII includes a point mutation involving a substitution of at positions H134 of SEQ ID NO:2. A preferred substitution includes: H134Q. Further preferred substitutions include H134G, H134Y, H134N, H134E, H134D, H134R and H134K.

[0086] Another exemplary recombinant factor VIII includes a point mutation involving a substitution of at positions M147 of SEQ ID NO:2. A preferred substitution includes: M147T. Further preferred substitutions include M147A, M147G, M147S and M147P.

[0087] Another exemplary recombinant factor VIII includes a point mutation involving a substitution of at positions L152 of SEQ ID NO:2. A preferred substitution includes: L152P. Further preferred substitutions include L152S, L152G and L152T.

[0088] Another exemplary recombinant factor VIII includes multiple substitutions of one or more amino acid residues at positions I86, A108, G132, M147, L152 of SEQ ID NO:2. The substitution(s) can include any permutation of mutations encompassing these five amino acid sites. Specific embodiments may include mutations at one or more substitution mutations selected from the group consisting of I86V, A108S, G132K, M147T, L152P. Further preferred embodiments include 2, 3, 4 or 5 substitutions, including any combination (or permutation) of substitutions selected from I86V, A108S, G132K, M147T and L152P.

[0089] Exemplary recombinant factor VIII mutants include point mutation(s) involving substitution(s) at one or more amino acid residues in SEQ ID NO:2 selected from the group consisting of I86, Y105, A108, D115, Q117, F129, G132, I4134, M147 and L152. The substitution(s) can include any permutation of mutations encompassing these ten amino acid sites. Specific embodiments may include mutations at one or more substitution mutations selected from the group consisting of I86V, Y105F, A108S, D115E, Q117H, F129L, G132K, H134Q, M147T and L152P. Further preferred embodiments include 2, 3, 4 or up to 9 substitutions, including any combination (or permutation) of substitutions selected from I86V, Y105F, A108S, D115E, Q117H, F129L, G132K, H134Q, M147T and L152P.

[0090] The nucleic acids encoding the above mentioned factor VIII substitutions are included in this invention and include all possible nucleic acids encoding the breadth of substitution mutants described herein.

[0091] Compared to wild type factor VIII in production (in cell lines or in vivo), the above described factor VIII mutants may exhibit increases in factor VIII secretion of between 5% to 10,000 fold, 10% to 2,000 fold, 50% up to 500 fold, 2 to 200 fold, 5 to 100 fold, 10 to 50 fold, at least 2 fold, at least 5 fold, at least 10 fold, at least 20 fold, at least 50 fold, at least 100 fold, at least 200 fold, at least 500 fold, at least 2,000 fold or at least 10,000 fold.

[0092] In certain embodiments, suitable mutant factor VIII sequences may be further modified to include, delete, or modify other factor VIII sequences to confer properties with regard to other attributes, including, without limitation, antigenicity, circulating half-life, protein secretion, affinity for factor IXa and/or factor X, altered factor VIII-inactivation

cleavage sites, stability of the activated factor VIII form, immunogenicity, and shelf-life.

[0093] In certain specific embodiments, the mutant factor VIII may be modified to produce a B-domain deleted (BDD) or "B-domainless" factor VIII product. FIG. 1 shows an exemplary BDD factor VIII embodiment containing amino acid residues 1-740 and 1690-2332 of SEQ ID NO:2. Preferably, the recombinant B-domainless factor VIII contains one or multiple substitutions at positions I86, Y105, A108, D115, Q117, F129, G132, H134, M147, L152 as described herein.

[0094] In one embodiment, a B-domainless recombinant factor VIII is produced, whereby the B-domain is replaced with a DNA linker segment and at least one codon is replaced with a codon encoding an amino acid residue having the same charge as a corresponding residue of porcine factor VIII (see, e.g., U.S. Patent Application Publication No. 2004/0197875 to Hauser, et al.).

[0095] In another embodiment, a B-domainless recombinant factor VIII is produced having a truncated factor IX intron 1 inserted in one or more locations (see, e.g., U.S. Pat. No. 6,800,461 to Negrier and U.S. Pat. No. 6,780,614 to Negrier). This recombinant factor VIII can be used for yielding higher production of the recombinant factor VIII in vitro as well as in a transfer vector for gene therapy (see, e.g., U.S. Pat. No. 6,800,461 to Negrier). In a particular embodiment, the recombinant factor VIII can be encoded by a nucleotide sequence having a truncated factor IX intron 1 inserted in two locations and having a promoter that is suitable for driving expression in hematopoietic cell lines, and specifically in platelets (see, e.g., U.S. Pat. No. 6,780,614 to Negrier).

[0096] A second example of a suitable mutant factor VIII that can be modified in accordance with the present invention is a chimeric human/animal factor VIII that contains one or more animal amino acid residues as substitution(s) for human amino acid residues that are responsible for the antigenicity of human factor VIII. In particular, animal (e.g., porcine) residue substitutions can include, without limitation, one or more of the following: R484A, R488G, P485A, L486S, Y487L, Y487A, S488A, S488L, R489A, R489S, R490G, L491S, P492L, P492A, K493A, G494S, V495A, K496M, H497L, L498S, K499M, D500A, F501A, P502L, L503M, L504M, P505A, G506A, E507G, L508M, L508A, M2199I, F2200L, L2252F, V2223A, K2227E and/or L2251 (U.S. Pat. No. 5,859,204 to Lollar, U.S. Pat. No. 6,770,744 to Lollar, and U.S. Patent Application Publication No. 2003/0166536 to Lollar). Preferably, the recombinant chimeric factor VIII contains one or multiple substitutions at positions I86, Y105, A108, D115, Q117, F129, G132, H134, M147 and L152 as described herein.

[0097] In a further embodiment, the mutant factor VIII is modified to confer greater stability of activated factor VIII by virtue of fused A2 and A3 domains. In particular, a factor VIII can be modified by substituting cysteine residues at positions 664 and 1826, (i.e., Y664C, T1826C) resulting in a mutant factor VIII forming a Cys664-Cys1826 disulfide bond covalently linking the A2 and A3 domains (Gale, et al., "An Engineered Interdomain Disulfide Bond Stabilizes Human Blood Coagulation Factor VIIIa," J. Thrombosis and Haemostasis 1(9):1966-1971 (2003)). Preferably, the recombinant fused domain (A2-A3) factor VIII contains one or multiple substitutions at positions I86, Y105, A108, D115, Q117, F129, G132, H134, M147 and L152 as described herein.

[0098] In a further embodiment, a mutant factor VIII in accordance with the present invention (e.g., containing one or

multiple substitutions at positions I86, Y105, A108, D115, Q117, F129, G132, H134, M147 and/or L152) is further modified to confer altered inactivation cleavage sites. For example, Arg336 or Arg562 may be substituted used to decrease the mutant factor VIII's susceptibility to cleavage enzymes that normally inactivate the wild type factor VIII (see, e.g., Amano, et al., "Mutation at Either Arg336 or Arg562 in Factor VIII is Insufficient for Complete Resistance to Activated Protein C (APC)-Mediated Inactivation: implications for the APC Resistance Test," *Thrombosis & Haemostasis* 79(3):557-63 (1998)).

[0099] In a further embodiment, a mutant factor VIII in accordance with the present invention (e.g., containing one or multiple substitutions at positions I86, Y105, A108, D115, Q117, F129, G132, H134, M147 and/or L152) is further modified to confer enhanced affinity for factor IXa (see, e.g., Fay, et al., "Factor VIIIa A2 Subunit Residues 558-565 Represent a Factor IXa Interactive Site," *J. Biol. Chem.* 269(32): 20522-7 (1994); Bajaj, et al., "Factor IXa: Factor VIIIa Interaction. Helix 330-338 of Factor IXa Interacts with Residues 558-565 and Spatially Adjacent Regions of the A2 Subunit of Factor VIIIa," *J. Biol. Chem.* 276(19):16302-9 (2001); and Lenting, et al., "The Sequence Glu1811-Lys1818 of Human Blood Coagulation Factor VIII Comprises a Binding Site for Activated Factor IX," *J. Biol. Chem.* 271(4):1935-40 (1996)) and/or factor X (see, e.g., Lapan, et al., "Localization of a Factor X Interactive Site in the A1 Subunit of Factor VIIIa," *J. Biol. Chem.* 272:2082-88 (1997)).

[0100] In yet another further embodiment, a mutant factor VIII in accordance with the present invention (e.g., containing one or multiple substitutions at positions I86, Y105, A108, D115, Q117, F129, G132, H134, M147 and/or L152) is further modified to further enhance secretion of factor VIII (see, e.g., Swaroop, et al., "Mutagenesis of a Potential Immunoglobulin-Binding Protein-Binding Site Enhances Secretion of Coagulation Factor VIII," *J. Biol. Chem.* 272(39): 24121-4 (1997)).

[0101] In a further embodiment, a mutant factor VIII in accordance with the present invention (e.g., containing one or multiple substitutions at positions I86, Y105, A108, D115, Q117, F129, G132, H134, M147 and/or L152) is further modified to confer increased circulating half-life. This may be achieved through various approaches, including, without limitation, by reducing interactions with heparan sulfate (Sarafanov, et al., "Cell Surface Heparan Sulfate Proteoglycans Participate in Factor VIII Catabolism Mediated by Low Density Lipoprotein Receptor-Related Protein," *J. Biol. Chem.* 276(15):11970-9 (2001)) and/or low-density lipoprotein receptor-related protein ("LRP") (Saenko, et al., "Role of the Low Density Lipoprotein-Related Protein Receptor in Mediation of Factor VIII Catabolism," *J. Biol. Chem.* 274 (53):37685-92 (1999); and "The Light Chain of Factor VIII Comprises a Binding Site for Low Density Lipoprotein Receptor-Related Protein," *J. Biol. Chem.* 274(34):23734-9 (1999)).

[0102] An eighth example of a suitable mutant factor VIII that can be modified in accordance with the present invention is a modified factor VIII encoded by a nucleotide sequence modified to code for amino acids within known, existing epitopes to produce a recognition sequence for glycosylation at asparagine residues (see, e.g., U.S. Pat. No. 6,759,216 to Lollar). Such modification can be useful escaping detection by existing inhibitory antibodies (low antigenicity factor VIII) and decreasing the likelihood of developing inhibitory

antibodies (low immunogenicity factor VIII). In one representative embodiment, the modified factor VIII is mutated to incorporate a consensus amino acid sequence for N-linked glycosylation, such as N-X-S/T (see U.S. Pat. No. 6,759,216 to Lollar).

[0103] A ninth example of a suitable mutant factor VIII that can be modified in accordance with the present invention is a modified factor VIII that is a procoagulant-active factor VIII having various mutations (see, e.g., U.S. Patent Application Publication No. 2004/0092442 to Kaufman, et al.). One example of this embodiment relates to a modified factor VIII that has been modified to (i) delete the von Willebrand factor binding site, (ii) add a mutation at Arg 740, and (iii) add an amino acid sequence spacer between the A2- and A3-domains, where the amino acid spacer is of a sufficient length so that upon activation, the procoagulant-active factor VIII protein becomes a heterodimer (see U.S. Patent Application Publication No. 2004/0092442 to Kaufman, et al.).

[0104] Further, the mutant factor VIII can be modified to take advantage of various advancements regarding recombinant coagulation factors generally (see, e.g., Saenko, et al., "The Future of Recombinant Coagulation Factors," *J. Thrombosis and Haemostasis* 1:922-930 (2003)).

[0105] The recombinant factor VIII of the present invention can be modified at position I86, Y105, A108, D115, Q117, F129, G132, H134, M147, L152, as well as be modified to be B-domainless, to be chimeric, to have fused A2-A3 domains, to have altered inactivation cleavage sites, to have enhanced factor IXa and/or factor X affinity, to have enhanced specific activity, to have an increased circulating half-life, to have mutant glycosylation sites, or to possess any two or more of such modifications in addition to the modifications at position (s) I86, Y105, A108, D115, Q117, F129, G132, H134, M147 and/or L152.

[0106] Another aspect of the present invention relates to a method of making a recombinant factor VIII having increased specific activity compared to that of a wild-type factor VIII. This method involves altering the amino acid sequence of a wild-type factor VIII to yield a recombinant factor VIII. Alteration of the amino acid sequence of the wild-type factor VIII can include, for example, introducing at least one point mutation in or near at least one calcium binding site of the wild-type factor VIII. Thereafter, using protein analysis techniques well-known in the art, a determination can be made as to whether the recombinant factor VIII has increased specific activity compared to that of the wild-type factor VIII.

[0107] The recombinant factor VIII is preferably produced in a substantially pure form. In a particular embodiment, the substantially pure recombinant factor VIII is at least about 80% pure, more preferably at least 90% pure, most preferably at least 95% pure, 98% pure, 99% pure or 99.9% pure. A substantially pure recombinant factor VIII can be obtained by conventional techniques well known in the art. Typically, the substantially pure recombinant factor VIII is secreted into the growth medium of recombinant host cells. Alternatively, the substantially pure recombinant factor VIII is produced but not secreted into growth medium. In such cases, to isolate the substantially pure recombinant factor VIII, the host cell carrying the recombinant plasmid is propagated, lysed by sonication, heat, or chemical treatment, and the homogenate is centrifuged to remove cell debris. The supernatant is then subjected to sequential ammonium sulfate precipitation. The fraction containing the substantially pure recombinant factor VIII is subjected to gel filtration in an appropriately sized

dextran or polyacrylamide column to separate the recombinant factor VIII. If necessary, a protein fraction (containing the substantially pure recombinant factor VIII) may be further purified by high performance liquid chromatography ("HPLC").

[0108] Another aspect of the present invention relates to an isolated nucleic acid molecule that encodes a recombinant mutant factor VIII as described herein. The isolated nucleic acid molecule encoding the recombinant mutant factor VIII can be an RNA or DNA. The nucleic acid codons can be further optimized for enhanced expression.

[0109] In one embodiment, the isolated nucleic acid molecule can have a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:2 as modified with one of the substitutions at positions identified in this invention (i.e., possessing one to three nucleotide substitutions within codon 86 (nt256-258), codon 105 (nt:313-315), codon 108 (nt:322-324), codon 115 (nt:343-345), codon 117 (nt:349-351), codon 129 (nt:385-387), codon 132 (nt:394-396), codon 134 (nt:400-402), codon 147 (nt:439-441) and/or codon 152 (nt:454-456) of SEQ ID NO:1 (the first 57 nucleotides are not counted since they encode the signal peptides). The isolated nucleic acid molecule may have one or multiple changes in these positions in any combination.

[0110] In another embodiment, the isolated nucleic acid molecule can have a nucleotide sequence encoding a B-domainless factor VIII of the type described above, as modified with one or multiple substitutions at position(s) I86, Y105, A108, D115, Q117, F129, G132, H134, M147 and/or L152.

[0111] In another embodiment, the isolated nucleic acid molecule can have a nucleotide sequence encoding a chimeric human/porcine of the type described above, as modified with one or multiple substitutions at position(s) I86, Y105, A108, D115, Q117, F129, G132, H134, M147 and/or L152.

[0112] In a further embodiment, the isolated nucleic acid molecule can have a nucleotide sequence encoding a fused A2-A3 domain factor VIII of the type described above, as modified with one or multiple substitutions at position(s) I86, Y105, A108, D115, Q117, F129, G132, H134, M147 and/or L152.

[0113] In another embodiment, the isolated nucleic acid molecule can have a nucleotide sequence encoding a factor VIII whose inactivation sites have been modified as described above, as further modified with one or multiple substitutions at position(s) I86, Y105, A108, D115, Q117, F129, G132, H134, M147 and/or L152.

[0114] In yet another embodiment, the isolated nucleic acid molecule can have a nucleotide sequence encoding a factor VIII whose affinity for factor IXa and/or factor X has been enhanced, along with one or multiple substitutions at position (s) I86, Y105, A108, D115, Q117, F129, G132, H134, M147 and/or L152.

[0115] In a still further embodiment, the isolated nucleic acid molecule can have a nucleotide sequence encoding a factor VIII whose affinity for various serum-binding proteins has been altered to increase its circulating half-life and further modified with one or multiple substitutions at position(s) I86, Y105, A108, D115, Q117, F129, G132, H134, M147 and/or L152.

[0116] In a further embodiment, the isolated nucleic acid molecule can have a nucleotide sequence encoding a factor VIII that has increased secretion in culture, as further modified

with one or multiple substitutions at position(s) I86, Y105, A108, D115, Q117, F129, G132, H134, M147 and/or L152.

[0117] In a further embodiment, the isolated nucleic acid molecule can have a nucleotide sequence encoding a factor VIII that possesses one or more non-naturally occurring glycosylation site(s) along with one or multiple substitutions at position(s) I86, Y105, A108, D115, Q117, F129, G132, H134, M147 and/or L152.

[0118] In yet another embodiment, the isolated nucleic acid molecule encodes a recombinant factor VIII that is modified with one or more substitutions at position(s) I86, Y105, A108, D115, Q117, F129, G132, H134, M147 and/or L152 and is further modified to possess any combination of the following modification: modified to be B-domainless, modified to be chimeric, modified to have fused A2-A3 domains, modified to have one or more altered inactivation cleavage site(s), modified to have enhanced factor IXa and/or factor X affinity, modified to have enhanced secretion, modified to have an increased circulating half-life, and modified to possess one or more non-naturally occurring glycosylation site(s).

[0119] Another aspect of the present invention relates to an expression vector for expressing the mutant factor VIII polynucleotides described herein. As used herein, the term "expression vector" refers to a viral or non-viral vector that comprise a polynucleotide encoding the novel peptide of the present invention in a form suitable for expression of the polynucleotide in a host cell. One type of non-viral vector is a "plasmid," which includes a circular double-stranded DNA loop into which additional DNA segments can be ligated. In the present specification, "plasmid" and "vector" can be used interchangeably as the plasmid is the most commonly used form of vector.

[0120] When preparing an expression vector, the transgene sequences may be inserted into a plasmid containing suitable bacterial sequences for replication in bacterial, as well as eukaryotic cells. Any convenient plasmid may be employed, which can include markers allowing for selection in a bacterium, and generally one or more unique, conveniently located restriction sites. The selection of a vector will depend on the preferred transformation technique and target host for transformation.

[0121] Expression vectors for expressing mutant factor VIII polypeptides include one or more regulatory sequences operably linked to the polynucleotide sequence to be expressed. It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, and the like. The expression vectors of the invention can be introduced into host cells to thereby produce the mutant factor VIII proteins described herein.

[0122] As used herein, the term "control sequences" or "regulatory sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The term "control/regulatory sequence" is intended to include promoters, enhancers and other expression control elements (e.g., polyadenylation signals). Control/regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cells and those which direct expression of the nucleotide sequence only in certain host cells (e.g., tissue-specific regulatory sequences).

[0123] A nucleic acid sequence is “operably linked” to another nucleic acid sequence when the former is placed into a functional relationship with the latter. For example, a DNA for a presequence or secretory leader peptide is operably linked to DNA for a polypeptide if it is expressed as a pre-protein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, “operably linked” means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

[0124] Suitable expression vectors for directing expression in mammalian cells generally include a promoter, as well as other transcription and translation control sequences known in the art. In certain embodiments, the mammalian expression vector is capable of directing expression of the polynucleotide preferentially in a particular cell type (e.g., tissue-specific regulatory elements are used to express the polynucleotide). Tissue-specific regulatory elements are known in the art and may include, for example, liver cell-specific promoters and/or enhancers (e.g., albumin promoter, α -1 antitrypsin promoter, apoE enhancer). Alternatively, a constitutive promoter (e.g., HCMV) active in virtually any cell type may be used.

[0125] In certain preferred embodiments, the expression vectors are viral vectors. Viral vectors typically have one or more viral genes removed and include a gene/promotor cassette inserted into a viral genome insertion site for insertion of exogenous transgenes, including the mutant factor VIII genes described herein. The necessary functions of the removed gene(s) may be supplied by cell lines which have been engineered to express the gene products of the early genes in trans. Exemplary viral vectors include, but are not limited to, adeno-associated viral (AAV) vectors, retroviral vectors, including lentiviral vectors, adenoviral vectors, herpes viral vectors, and alphavirus vectors. Other viral vectors include astrovirus, coronavirus, orthomyxovirus, papovavirus, paramyxovirus, parvovirus, picornavirus, poxvirus, togavirus viral vectors and the like. The viral vector may comprise any suitable nucleic acid construct, such as a DNA or RNA construct and may be single stranded, double stranded, or duplexed.

[0126] Once the DNA construct of the present invention has been prepared, it is ready to be incorporated into a host cell. Accordingly, another aspect of the present invention relates to a method of making a recombinant cell comprising a factor VIII nucleic acid. Basically, this entails introducing the DNA construct into cells via transformation, transduction, electroporation, calcium phosphate precipitation, liposomes and the like and selecting for cells that have incorporated the DNA episomally or integrated into the host genome.

[0127] Thus, a further aspect of the present invention relates to a host cell including an isolated nucleic acid molecule encoding the recombinant factor VIII of the present invention. The host cell can contain the isolated nucleic acid molecule as a DNA molecule in the form of an episomal plasmid or stably integrated into the host cell genome. Further, the host cell can constitute an expression system for producing the recombinant mutant factor VIII protein. Suit-

able host cells can be, without limitation, animal cells (e.g., baby hamster kidney (“BHK”) cells), Chinese hamster ovary cells (“CHO”), bacterial cells (e.g., *E. coli*), insect cells (e.g., Sf9 cells), fungal cells, yeast cells (e.g., *Saccharomyces* or *Schizosaccharomyces*), plant cells (e.g., *Arabidopsis* or tobacco cells), algal cells and the like.

[0128] Another aspect of the present invention relates to a method of making a recombinant factor VIII of the present invention. This method involves growing a host cell of the present invention under conditions whereby the host cell expresses the recombinant factor VIII. The recombinant factor VIII is then isolated. In one embodiment, the host cell is grown in vitro in a growth medium. In a particular embodiment, suitable growth media can include, without limitation, a growth medium containing a von Willebrand Factor (referred to herein as “VWF”). In this embodiment, the host cell can contain a transgene encoding a VWF or the VWF can be introduced to the growth medium as a supplement. VWF in the growth medium will allow for greater expression levels of the recombinant factor VIII. Once the recombinant factor VIII is secreted into the growth medium, it can then be isolated from the growth medium using techniques well-known by those of ordinary skill in the relevant recombinant DNA and protein arts (including those described herein). In another embodiment, the method of making the recombinant factor VIII of the present invention further involves disrupting the host cell prior to isolation of the recombinant factor VIII. In this embodiment, the recombinant factor VIII is isolated from cellular debris.

[0129] When recombinantly produced, the factor VIII protein or polypeptide (or fragment or mutant thereof) is expressed in a recombinant host cell, typically, although not exclusively, a eukaryote. In certain preferred embodiments, eukaryotic host cells, such as mammalian cells, are used to produce mutant factor VIII polypeptides as described herein. Mammalian cells suitable for carrying out the present invention include, among others: COS (e.g., ATCC No. CRL 1650 or 1651), BHK (e.g., ATCC No. CRL 6281), CHO (ATCC No. CCL 61), HeLa (e.g., ATCC No. CCL 2), 293 (ATCC No. 1573), CHOP, and NS-1 cells.

[0130] Another aspect of the present invention relates to a method for treating patient with a factor VIII deficiency. In one embodiment, this involves administering to a patient in need thereof a recombinant mutant factor VIII (as described herein) in an amount effective for treating the factor VIII deficiency.

[0131] In a particular embodiment, the recombinant factor VIII, alone, or in the form of a pharmaceutical composition (i.e., in combination with stabilizers, delivery vehicles, and/or carriers) is infused into patients intravenously according to the same procedure that is used for infusion of human or animal factor VIII. A suitable effective amount of the recombinant factor VIII can include, without limitation, between about 10 to about 500 units/kg body weight of the patient.

[0132] In another embodiment, a method for treating patient with a factor VIII deficiency comprises administering to a patient in need thereof a pharmaceutical composition comprising an expression vector encoding a mutant factor VIII, or a functional fragments thereof, in an amount effective for treating the factor VIII deficiency. In certain embodiments, the recombinant factor VIII can be administered by transplanting cells genetically engineered to produce the recombinant factor VIII, typically via implantation of a device containing such cells. Such transplantation typically

involves using recombinant dermal fibroblasts, a non-viral approach (Roth, et al., *New Engl. J. Med.* 344:1735-1742 (2001)).

[0133] Administration of FVIII-encoding expression vectors to factor VIII deficient patients can result in sufficient expression of FVIII polypeptide to functionally reconstitute the coagulation cascade. The expression vector(s) may be administered alone or in combination with other therapeutic agents in a pharmaceutically acceptable or biologically compatible composition.

[0134] The FVIII-encoding polynucleotide can be employed as a single chain molecule containing both heavy and light chain portions (FIG. 10) or split into two or multiple molecules (e.g., heavy and light chain; FIG. 11) in viral or non-viral vectors for delivery into host cells of the patient.

[0135] In a preferred embodiment of the invention, the expression vector comprising nucleic acid sequences encoding the mutant FVIII mutants is a viral vector. Viral vectors which may be used in the present invention include, but are not limited to, adenoviral vectors (with or without tissue specific promoters/enhancers), adeno-associated virus (AAV) vectors of multiple serotypes (e.g., AAV-1 to AAV-12, and others) and hybrid AAV vectors, lentivirus vectors and pseudo-typed lentivirus vectors [e.g., Ebola virus, vesicular stomatitis virus (VSV), and feline immunodeficiency virus (FIV)], herpes simplex virus vectors, vaccinia virus vectors, retroviral vectors, lentiviral vectors, non-viral vectors and others.

[0136] In certain preferred embodiments, methods are provided for the administration of a viral vector comprising nucleic acid sequences encoding a mutant FVIII, or a functional fragment thereof involve the use of AAV vectors or lentiviral vectors. Most preferably, only the essential parts of vector e.g., the ITR and LTR elements, respectively are included. Direct delivery of vectors or ex-vivo transduction of human cells and followed by infusion into the body will result in expression of mutant FVIII thereby exerting a beneficial therapeutic effect on hemostasis by enhancing pro-coagulation activity.

[0137] Recombinant AAV and lentiviral vectors have found broad utility for a variety of gene therapy applications. Their utility for such applications is due largely to the high efficiency of in vivo gene transfer achieved in a variety of organ contexts. AAV and lentiviral particles may be used to advantage as vehicles for effective gene delivery. Such virions possess a number of desirable features for such applications, including tropism for dividing and non-dividing cells. Early clinical experience with these vectors also demonstrated no sustained toxicity and immune responses were minimal or undetectable. AAV are known to infect a wide variety of cell types in vivo and in vitro by receptor-mediated endocytosis or by transcytosis. These vector systems have been tested in humans targeting retinal epithelium, liver, skeletal muscle, airways, brain, joints and hematopoietic stem cells. It is likely that non-viral vectors based on plasmid DNA or minicircles will be also suitable gene transfer vectors for a large gene as that encoding FVIII.

[0138] It is desirable to introduce a vector that can provide, for example, sufficient expression of a desired gene and minimal immunogenicity. Improved AAV and lentiviral vectors and methods for producing such vectors have been described in detail in a number of references, patents, and patent applications, including Wright J. F. (*Hum Gene Ther* 20:698-706, 2009) which describes the production of clinical grade vec-

tors. Lentiviral vector can be produced at CHOP and the other vectors are available through the Lentivirus vector production core laboratory by NHLBI Gene Therapy Resource Program (GTRP)-Lentivirus Vector Production Core Laboratory.

[0139] For some applications, an expression construct may further comprise regulatory elements which serve to drive expression in a particular cell or tissue type. Such regulatory elements are known to those of skill in the art and discussed in depth in Sambrook, et al. (1989) and Ausubel, et al. (1992). The incorporation of tissue specific regulatory elements in the expression constructs of the present invention provides for at least partial tissue tropism for the expression of the mutant FVIII or functional fragments thereof. For example, nucleic acid sequences encoding mutant FVIII under the control of a cytomegalovirus (CMV) promoter can be employed for skeletal muscle expression or the hAAT-ApoE and others for liver specific expression. Hematopoietic specific promoters in lentiviral vectors may also be used to advantage in the methods of the present invention.

[0140] In a preferred embodiment, the mutant FVIII sequence is provided as a component of a viral vector packaged in a capsid. In a particularly preferred embodiment, an AAV vector is used for in vivo delivery of the mutant FVIII (Gnatenko, et al., *Br. J. Haematol.* 104:27-36 (1999)). In this case, the AAV vector includes at least one mutant FVIII and associated expression control sequences for controlling expression of the mutant FVIII sequence. As shown in FIGS. 10 and 11, exemplary AAV vectors for expressing mutant FVIII sequences may include promoter-enhancer regulatory regions for FVIII expression and cis-acting ITRs functioning to enable promote replication and packaging of the mutant FVIII nucleic acids into AAV capsids and integration of the mutant FVIII nucleic acid into the genome of a target cell. Preferably, the AAV vector has its rep and cap genes deleted and replaced by the mutant hFVIII sequence and its associated expression control sequences. As shown in FIGS. 10 and 11, the mutant FVIII sequence is typically inserted adjacent to one or two (i.e., flanked by) AAV TRs or TR elements adequate for viral replication. Other regulatory sequences suitable for facilitating tissue-specific expression of the mutant hFVIII sequence in the target cell may also be included.

[0141] The viral capsid component of the packaged viral vectors may be a parvovirus capsid. AAV Cap and chimeric capsids are preferred. Examples of suitable parvovirus viral capsid components are capsid components from the Parvoviridae family, such as an autonomous parvovirus or a dependovirus. For example, the viral capsid may be an AAV capsid (e.g., AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11 or AAV12 capsid; one skilled in the art would know there are likely other variants not yet identified that perform the same or similar function), or may include components from two or more AAV capsids. A full complement of AAV Cap proteins includes VP1, VP2, and VP3. The ORF comprising nucleotide sequences encoding AAV VP capsid proteins may comprise less than a full complement AAV Cap proteins or the full complement of AAV Cap proteins may be provided.

[0142] One or more of the AAV Cap proteins may be a chimeric protein, including amino acid sequences AAV Caps from two or more viruses, preferably two or more AAVs, as described in Rabinowitz et al., U.S. Pat. No. 6,491,907. For example, the chimeric virus capsid can include an AAV1 Cap protein or subunit and at least one AAV2 Cap or subunit. The

chimeric capsid can, for example, include an AAV capsid with one or more B19 Cap subunits, e.g., an AAV Cap protein or subunit can be replaced by a B19 Cap protein or subunit. For example, the Vp3 subunit of the AAV capsid can be replaced by the Vp2 subunit of B19.

[0143] Packaging cells may be cultured to produce packaged viral vectors of the invention. The packaging cells may include heterologous (1) viral vector function(s), (2) packaging function(s), and (3) helper function(s). The viral vector functions typically include a portion of a parvovirus genome, such as an AAV genome, with rep and cap deleted and replaced by the mutant FVIII sequence and its associated expression control sequences as described above.

[0144] In certain embodiments, the viral vector functions may suitably be provided as duplexed vector templates, as described in U.S. Patent Publication No. 2004/0029106 to Samulski et al. Duplexed vectors are dimeric self-complementary (sc) polynucleotides (typically, DNA). For example, the DNA of the duplexed vectors can be selected so as to form a double-stranded hairpin structure due to intrastrand base pairing. Both strands of the duplexed DNA vectors may be packaged within a viral capsid. The duplexed vector provides a function comparable to double-stranded DNA virus vectors and can alleviate the need of the target cell to synthesize complementary DNA to the single-stranded genome normally encapsidated by the virus.

[0145] The TR(s) (resolvable and non-resolvable) selected for use in the viral vectors are preferably AAV sequences (from any AAV serotype). Resolvable AAV ITRs need not have a wild-type TR sequence (e.g., a wild-type sequence may be altered by insertion, deletion, truncation or missense mutations), as long as the TR mediates the desired functions, e.g., virus packaging, integration, and/or provirus rescue, and the like. The TRs may be synthetic sequences that function as AAV inverted terminal repeats, such as the “double-D sequence” as described in U.S. Pat. No. 5,478,745 to Samulski et al. Typically, but not necessarily, the TRs are from the same parvovirus, e.g., both TR sequences are from AAV2.

[0146] The packaging functions include capsid components. The capsid components are preferably from a parvoviral capsid, such as an AAV capsid or a chimeric AAV capsid function. Examples of suitable parvovirus viral capsid components are capsid components from the family Parvoviridae, such as an autonomous parvovirus or a Dependovirus. For example, the capsid components may be selected from AAV capsids, e.g., AAV1-AAV12 and other novel capsids as yet unidentified or from non-human primate sources. Capsid components may include components from two or more AAV capsids.

[0147] In certain embodiments, one or more of the VP capsid proteins may comprise chimeric proteins, comprising amino acid sequences from two or more viruses, preferably two or more AAVs, as described in Rabinowitz et al., U.S. Pat. No. 6,491,907. For example, the chimeric virus capsid can include a capsid region from an adeno-associated virus (AAV) and at least one capsid region from a B19 virus. The chimeric capsid can, for example, include an AAV capsid with one or more B19 capsid subunits, e.g., an AAV capsid subunit can be replaced by a B19 capsid subunit. For example, the VP1, VP2 or VP3 subunit of the AAV capsid can be replaced by the VP1, VP2 or VP3 subunit of B19. As another example, the chimeric capsid may include an AAV type 2 capsid in which the type 2 VP1 subunit has been replaced by the VP1 subunit from an AAV type 1, 3, 4, 5, or 6 capsid,

preferably a type 3, 4, or 5 capsid. Alternatively, the chimeric parvovirus has an AAV type 2 capsid in which the type 2 VP2 subunit has been replaced by the VP2 subunit from an AAV type 1, 3, 4, 5, or 6 capsid, preferably a type 3, 4, or 5 capsid. Likewise, chimeric parvoviruses in which the VP3 subunit from an AAV type 1, 3, 4, 5 or 6 (more preferably, type 3, 4 or 5) is substituted for the VP3 subunit of an AAV type 2 capsid are preferred. As a further alternative, chimeric parvoviruses in which two of the AAV type 2 subunits are replaced by the subunits from an AAV of a different serotype (e.g., AAV type 1, 3, 4, 5 or 6) are preferred. In exemplary chimeric parvoviruses according to this embodiment, the VP1 and VP2, or VP1 and VP3, or VP2 and VP3 subunits of an AAV type 2 capsid are replaced by the corresponding subunits of an AAV of a different serotype (e.g., AAV type 1, 3, 4, 5 or 6). Likewise, in other preferred embodiments, the chimeric parvovirus has an AAV type 1, 3, 4, 5 or 6 capsid (preferably the type 2, 3 or 5 capsid) in which one or two subunits have been replaced with those from an AAV of a different serotype, as described above for AAV type 2.

[0148] The packaged viral vector generally includes the mutant FVIII sequence and expression control sequences flanked by TR elements sufficient to result in packaging of the vector DNA and subsequent expression of the mutant FVIII sequence in the transduced cell. The viral vector functions may, for example, be supplied to the cell as a component of a plasmid or an amplicon. The viral vector functions may exist extrachromosomally within the cell line and/or may be integrated into the cells' chromosomal DNA.

[0149] In a preferred embodiment, the mutant FVIII described herein are used for gene therapy of FVIII associated disorders, such as hemophilia A. In this case, expression of the mutant FVIII transgene can enhance clotting in a subject otherwise vulnerable to uncontrolled bleeding due to factor VIII deficiency (e.g., intraarticular, intracranial, or gastrointestinal hemorrhage), including hemophiliacs who have developed antibodies to human factor VIII. The target cells of the vectors are cells capable of expressing polypeptides with FVIII activity, such as those of the hepatic system of a mammal, endothelial cells and other cells with the proper cellular machinery to process the precursor to yield protein with FVIII activity.

[0150] In particular embodiments, the present invention provides a pharmaceutical composition comprising a vector of the present invention including a modified gene of FVIII in a pharmaceutically-acceptable carrier and/or other medicinal agents, pharmaceutical agents, carriers, adjuvants, diluents, etc.

[0151] The treatment dosages of recombinant factor VIII that should be administered to a patient in need of such treatment will vary depending on the severity of the factor VIII deficiency. Generally, dosage level is adjusted in frequency, duration, and units in keeping with the severity and duration of each patient's bleeding episode. Accordingly, the recombinant factor VIII is included in a pharmaceutically acceptable carrier, delivery vehicle, or stabilizer in an amount sufficient to deliver to a patient a therapeutically effective amount of the protein to stop bleeding, as measured by standard clotting assays.

[0152] Factor VIII is classically defined as that substance present in normal blood plasma that corrects the clotting defect in plasma derived from individuals with hemophilia A. The coagulant activity in vitro of purified and partially-purified forms of factor VIII is used to calculate the dose of

recombinant factor VIII for infusions in human patients and is a reliable indicator of activity recovered from patient plasma and of correction of the in vivo bleeding defect. There are no reported discrepancies between standard assay of novel factor VIII molecules in vitro and their behavior in the dog infusion model or in human patients, according to Lusher, et al., *New Engl. J. Med.* 328:453-459 (1993); Pittman, et al., *Blood* 79:389-397 (1992); and Brinkhous, et al., *Proc. Natl. Acad. Sci.* 82:8752-8755 (1985).

[0153] Usually, the desired plasma factor VIII activity level to be achieved in the patient through administration of the recombinant factor VIII is in the range of 30-200% of normal. In one embodiment, administration of the therapeutic recombinant factor VIII is given intravenously at a preferred dosage in the range from about 5 to 500 units/kg body weight, and particularly in a range of 10-100 units/kg body weight, and further particularly at a dosage of 20-40 units/kg body weight; the interval frequency is in the range from about 8 to 24 hours (in severely affected hemophiliacs); and the duration of treatment in days is in the range from 1 to 10 days or until the bleeding episode is resolved. See, e.g., Roberts, H. R., and M. R. Jones, "Hemophilia and Related Conditions—Congenital Deficiencies of Prothrombin (Factor II, Factor V, and Factors VII to XII)," Ch. 153, 1453-1474, 1460, in *Hematology*, Williams, W. J., et al., ed. (1990). Patients with inhibitors may require a different amount of recombinant factor VIII than their previous form of factor VIII. For example, patients may require less recombinant factor VIII because of its higher specific activity than the wild-type VIII and its decreased antibody reactivity. As in treatment with human or plasma-derived factor VIII, the amount of therapeutic recombinant factor VIII infused is defined by the one-stage factor VIII coagulation assay and, in selected instances, in vivo recovery is determined by measuring the factor VIII in the patient's plasma after infusion. It is to be understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed recombinant factor VIII.

[0154] Treatment can take the form of a single intravenous administration of the recombinant factor VIII or periodic or continuous administration over an extended period of time, as required. Alternatively, therapeutic recombinant factor VIII can be administered subcutaneously or orally with liposomes in one or several doses at varying intervals of time.

[0155] For injection, the carrier will typically be a liquid. For other methods of administration, the carrier may be either solid or liquid. For inhalation administration, the carrier will be respirable, and will preferably be in solid or liquid particulate form. As an injection medium, it is preferred to use water that contains the additives usual for injection solutions, such as stabilizing agents, salts or saline, and/or buffers.

[0156] Exemplary pharmaceutically acceptable carriers include sterile, pyrogen-free water and sterile, pyrogen-free, phosphate buffered saline. Physiologically-acceptable carriers include pharmaceutically-acceptable carriers. Pharmaceutically acceptable carriers are those which are that is not biologically or otherwise undesirable, i.e., the material may be administered to a subject without causing undesirable biological effects which outweigh the advantageous biological effects of the material. A pharmaceutical composition

may be used, for example, in transfection of a cell *ex vivo* or in administering a viral vector or cell directly to a subject.

[0157] Recombinant virus vectors comprising the modified gene of FVIII are preferably administered to the cell in a biologically-effective amount. If the virus vector is administered to a cell in vivo (e.g., the virus is administered to a subject as described below), a biologically-effective amount of the virus vector is an amount that is sufficient to result in transduction and expression of the transgene in a target cell.

[0158] The cells transduced with a viral vector are preferably administered to the subject in a "therapeutically-effective amount" in combination with a pharmaceutical carrier. Those skilled in the art will appreciate that the therapeutic effects need not be complete or curative, as long as some benefit is provided to the subject.

[0159] Dosages of the cells to administer to a subject will vary upon the age, condition and species of the subject, the type of cell, the nucleic acid being expressed by the cell, the mode of administration, and the like. Typically, at least about 10^2 to about 10^8 , preferably about 10^3 to about 10^8 cells, will be administered per dose. Preferably, the cells will be administered in a therapeutically-effective amount.

[0160] Administration of the vector to a human subject or an animal in need thereof can be by any means known in the art for administering virus vectors. Exemplary modes of administration include rectal, transmucosal, topical, transdermal, inhalation, parenteral (e.g., intravenous, subcutaneous, intradermal, intramuscular, and intraarticular) administration, and the like, as well as direct tissue or organ injection, alternatively, intrathecal, direct intramuscular, intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Alternatively, one may administer the virus in a local rather than systemic manner, for example, in a depot or sustained-release formulation.

[0161] In other preferred embodiments, the inventive vector comprising the mutant FVIII gene is administered intramuscularly, more preferably by intramuscular injection or by local administration. The vectors disclosed herein may be administered to the lungs of a subject by any suitable means, but are preferably administered by administering an aerosol suspension of respirable particles comprised of the inventive parvovirus vectors, which the subject inhales. The respirable particles may be liquid or solid. Aerosols of liquid particles comprising the inventive parvovirus vectors may be produced by any suitable means, such as with a pressure-driven aerosol nebulizer or an ultrasonic nebulizer, as is known to those of skill in the art. See, e.g., U.S. Pat. No. 4,501,729.

[0162] Dosages of the virus vector with the mutant FVIII gene will depend upon the mode of administration, the disease or condition to be treated, the individual subject's condition, the particular viral vector, and the gene to be delivered, and can be determined in a routine manner. Exemplary doses for achieving therapeutic effects are virus titers of at least about 10^5 , 10^6 , 10^7 , 10^8 , 10^9 , 10^{10} , 10^{11} , 10^{12} , 10^{13} , 10^{14} , 10^{15} transducing units or more, preferably about 10^8 - 10^{13} transducing units, yet more preferably 10^{12} transducing units. The mutant FVIII genes may be administered as components of a DNA molecule having regulatory elements appropriate for expression in the target cells. The mutant FVIII genes may be administered as components of viral plasmids, such as rAAV vectors. Viral particles may be administered as viral particles

alone, whether as an in vivo direct delivery to the portal vasculature or as an ex vivo treatment comprising administering the vector viral particles in vitro to cells from the animal receiving treatment followed by introduction of the transduced cells back into the donor.

[0163] The present invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents, and published patent applications cited throughout this application are incorporated herein by reference.

EXAMPLE 1

Construction, Expression and Purification of B-Domainless Factor VIII Mutants

[0164] Plasmid pAAV-CB-F8 carries a B-domainless human factor VIII (hF8) cDNA under the control of a CB promoter (beta-actin promoter with a CMV enhancer). This plasmid, consistent with the construction shown in FIG. 10, was used as template for making various hF8 mutants. A hF8 cDNA fragment encoding the substitution mutations I86V, Y105F, A108S, D115E, Q117H, F129L, G132K, H134Q, M147T and L152P was synthesized chemically and used to replace the corresponding region of pAAV-CB-F8. The resulting plasmid (pAAV-CB-F8-X10) expresses a mutant factor VIII protein with the above 10 mutations (F8-X10; SEQ ID NO: 3).

[0165] A factor VIII cDNA fragment encoding the substitution mutations I86V, A108S, G132K, M147T, L152P was synthesized chemically and used to replace the corresponding region of pAAV-CB-F8. The resulting plasmid (pAAV-CB-F8-X5) expresses a mutant factor VIII protein with the above 5 mutations (F8-X5; SEQ ID NO:4).

[0166] Site-directed mutagenesis was used to introduce individual mutations corresponding to I86, Y105, A108, D115, Q117, F129, G132, H134, M147, L152 of human factor VIII in the pAAV-CB-F8 plasmid. The resulting plasmids include pAAV-CB-F8-I86V, pAAV-CB-F8-Y105F, pAAV-CB-F8-A108S, pAAV-CB-F8-D115E, pAAV-CB-F8-Q117H, pAAV-CB-F8-F129L, pAAV-CB-F8-G132K, pAAV-CB-F8-H134Q and pAAV-CB-F8-M147T, pAAV-CB-F8-L152P.

[0167] Site-directed mutagenesis was also used to revert back individual mutations in amino acids 86, 105, 108, 115, 117, 129, 132, 134, 147, 152 of hF8-X10 in the pAAV-CB-F8-X10 plasmid to wild-type human amino acids. The resulting plasmids include pAAV-CB-F8-X10-V86I, pAAV-CB-F8-X10-F105Y, pAAV-CB-F8-X10-5108A, pAAV-CB-F8-X10-E115D, pAAV-CB-F8-X10-H117Q, pAAV-CB-F8-X10-L129F, pAAV-CB-F8-X10-K132G, pAAV-CB-F8-X10-Q134H, pAAV-CB-F8-X10-T147M, pAAV-CB-F8-X10-P152L. These mutants were tested as described Example 4 (FIG. 7) below.

[0168] A set of degenerated oligonucleotides with NNN corresponding to G132 position was used to create all 19 mutations corresponding to G132. The resulting factor VIII mutant plasmids include pAAV-CB-F8-G132A, pAAV-CB-F8-G132I, . . . pAAV-CB-F8-G132V etc. The last letter indicates the amino acid substitution at that particular position. A similar strategy can be used to generate other substitutions in accordance with the present invention.

[0169] The above described plasmids contain an AAV-ITR and can be used to generate AAV vectors. A liver specific promoter can be used to replace the CB promoter to reduce

the size of the expression cassette and to improve vector packaging and expression in the liver. Other tissue specific promoter can also be used as well.

[0170] Sequences between the FVIII heavy chain and the FVIII stop codon were removed from the FVIII expression vectors. The resulting heavy chain (HC) mutants contain the first 745 amino acid of FVIII and lack B domain (BDD) and light chain (LC) sequences. The plasmid pAAV-CB-HC-X10 contains mutations corresponding to I86V, Y105F, A108S, D115E, Q117H, F129L, G132K, H134Q, M147T and L152P in the heavy chain. The plasmid pAAV-CB-HC-X5 contains mutations corresponding to I86V, A108S, G132K, M147T, L152P in the heavy chain. The plasmid pAAV-CB-HC-G132K contains the G132K mutation in the heavy chain. Alternatively, acidic region 3 (a3) can be added to these constructs to obtain HC mutants in an HC1690 background.

EXAMPLE 2

Comparison of Different Factor VIII Mutants for Secretion in Tissue Culture Cells

[0171] Plasmids pAAV-CB-hBDD-F8 (wt), pAAV-CB-hBDD-F8-X10, pAAV-CB-hBDD-F8-X5 and pAAV-CB-hBDD-F8-G312K were separately transfected in BHK cells (panel A) or 293 cells (panel B). Secreted F8 in the media was harvested and assayed by aPTT assay at 48 hours post transfection. The expression/secretion by wild type human BDD-F8(hBDD) was set as 100%. As shown in FIG. 7, the hF8 mutants were secreted at about 2-8.5 fold higher expression levels than the wild type hF8.

EXAMPLE 3

Comparison of Human Factor VIII Mutant Secretion In Vivo

[0172] Plasmids pAAV-CB-hBDDF8 (B-domain deleted (BDD) wt hF8), pAAV-CB-hBDDF8-X10 (hF8-BDD with 10 substitutions; SEQ ID NO:3), pAAV-CB-hBDD-F8-X5 (hF8 with 5 substitutions; SEQ ID NO:4) and pAAV-CB-hBDD-F8-G312K (hF8 with G132K substitution) were separately injected to Blab/c and F8 double knock-out mice. Secreted F8 in the blood was collected and assayed by aPTT assay at 48 hours post injection. The expression/secretion by wild type human F8 (hBDD-F8) was set as 100%. All mutants described here outperform the wild type factor VIII. As shown in FIG. 8, the hF8 mutants were secreted at about 1.2-5.2 fold higher expression levels than the wild type hF8.

EXAMPLE 4

Comparison of Different Factor VIII Mutants in Secretion

[0173] Plasmids encoding amino acids in hBDD-F8-X10 were modified to revert back the mutant substitutions to their corresponding wild type amino acids as indicated in the table. For example, hBDD-F8-X10-V86I means that "V86" in hBDD-F8-X10 was changed back to "I". In hBDD-F8-X10-6p, 6 of 10 mutant amino acids in hBDD-F8-X10 were reverted back to their corresponding wild type amino acids. The above plasmids were separately transfected in 293 cells. Secreted F8 in the media was harvested and assayed by aPTT assay at 48 hours post transfection. The expression/secretion by hBDD-F8-X10 was set as 100%. As shown in FIG. 9, all of

the revertants exhibited reduced expression and secretion as compared to the hBDD-F8-X10 mutant.

EXAMPLE 4

Comparison of Different Factor VIII Mutants in Secretion

[0174] AAV8 vector AAV-apoEhAAT-hF8HC1690 (carrying human factor VIII heavy chain aa#1-745 and a3 sequence, apoEhAAT: human alpha one antitrypsin promoter with apo E enhancer) and AAV-apoEhAAT-hF8-HC-X10 (hF8HC with 10 substitutions) were each separately injected in F8 knock-out mice with a light chain expression vector (dose: each vector 1E11 particles/mouse). Secreted F8 in the blood was collected and assayed by aPTT assay the indicated times. The expression/secretion by wild type human F8 heavy chain (hF8-HC1690) was set as 100%. The hF8-HC1690-X10 mutant described here outperform the wild type heavy chain expression. As shown in FIG. 10, the hF8-HC1690-X10 mutant was secreted at about 6-20 fold higher expression levels compared to wild type hF8HC.

EXAMPLE 6

Comparison of G132 Factor VIII Heavy Chain Mutants in Secretion in 293 Cells

[0175] Plasmid pAAV-CB-hF8-HC1690 (carrying human factor VIII heavy chain aa#1-745 and a3 sequence; see FIG. 1) or plasmids with indicated substitutions at G132 were transfected in 293 cells with an F8 light chain (LC) expression plasmid. Secreted F8 in the media was collected and assayed by aPTT assay at 48 hours post transfection. The expression/secretion by wild type human F8 heavy chain (hF8-HC1690) was set as 100%. As shown in FIG. 11, the F8 G132HC1690 mutants were secreted at about 1.4-3.4 fold higher expression levels compared to wild type hF8HC.

EXAMPLE 7

Comparison of Different Factor VIII Heavy Chain Mutants in Expression/Secretion In Vivo

[0176] Plasmids pAAV-CB-hF8-HC1690 (carrying human factor VIII heavy chain aa#1-745 and acidic region 3 (a3) sequence; see FIGS. 1, 6), pAAV-CB-hF8-HC1690-X10 (hF8 heavy chain with 10 substitutions; SEQ ID NO:5), pAAV-CB-hF8-HC1690-X5 (hF8 heavy chain with 5 substitutions along with a3 sequence; SEQ ID NO:6) and pAAV-CB-hF8-HC1690-G312K (hF8 heavy chain with G132K substitution and a3 sequence) were separately injected in Blab/c and F8 double knock-out mice along with a light chain expression plasmid. Secreted F8 in the blood was collected and assayed by aPTT assay at 48 hours post injection. The expression/secretion by wild type human F8 heavy chain (hF8-HC1690) was set as 100%. As shown in FIG. 12, the hF8 mutants were secreted at about 2.5-4.5 fold higher expression levels than the wild type hF8HC.

EXAMPLE 8

Comparison of L152 Factor VIII Heavy Chain Mutants in Secretion in 293 Cells

[0177] Plasmid pAAV-CB-hF8-HC1690 (carrying human factor VIII heavy chain aa#1-745 and a3 sequence; see FIGS.

1, 6) or plasmids with indicated substitutions at L152 were separately transfected in 293 cells with a hF8 light chain expression plasmid. Secreted hF8 in the media was collected and assayed by aPTT assay at 48 hours post transfection. The expression/secretion by wild type human F8 heavy chain (hF8-HC1690) was set as 100%. The hF8-L152HC mutants are identified by their specific amino acid substitutions in the Figure. As shown in FIG. 13, the hF8-L152HC mutants were secreted at about 1.4-3.5 fold higher expression levels compared to wild type hF8HC.

EXAMPLE 9

Comparison of A108 Factor VIII Heavy Chain Mutants in Secretion in 293 Cells

[0178] Plasmid pAAV-CB-hF8-HC1690 (carrying human factor VIII heavy chain aa#1-745 and a3 sequence; see FIG. 1) or plasmids with indicated substitutions at A108 were separately transfected in 293 cells with a hF8 light chain expression plasmid. Secreted hF8 in the media was collected and assayed by aPTT assay at 48 hours post transfection. The hF8-A108HC mutants are identified by their specific amino acid substitutions in the Figure. The expression/secretion by wild type human F8 heavy chain (hF8-HC1690) was set as 100%. As shown in FIG. 14, the majority of hF8-A108HC mutants were secreted at higher expression levels compared to wild type hF8HC.

EXAMPLE 10

Comparison of M147 Factor VIII Heavy Chain Mutants in Secretion in 293 Cells

[0179] Plasmid pAAV-CB-hF8-HC1690 (carrying human factor VIII heavy chain aa#1-745 and a3 sequence; see FIG. 1) or plasmids with indicated substitutions at M147 were separately transfected in 293 cells with a hF8 light chain expression plasmid. Secreted hF8 in the media was collected and assayed by hF8 ELISA at 48 hours post transfection. The hF8-M147HC mutants are identified by their specific amino acid substitutions in the Figure. The expression/secretion by wild type human F8 heavy chain (hF8-HC1690) was set as 100%. As shown in FIG. 15, the majority of hF8-M147HC mutants were secreted at higher expression levels compared to wild type hF8HC.

EXAMPLE 11

Neutralizing Antibodies Against 5 Mutated Amino Acids Were not Detected in the F8-/- Rat Model

[0180] F8-/- rats in a WAG/RijYcb background with single mutation in the A1 domain (Leu176Pro) were administered AAV-TTR-hF8-X10 at 1×10^{12} viral particles/per rat. Of 3 injected rats, two were confirmed to develop rat anti-human factor VIII inhibitory antibodies against factor VIII as determined by a Bethesda assay. Panel A is representative of inhibitor levels in rat plasma at week 8. Panel B shows the activity of F8 remains in the supernatant after antibody absorption. To determine whether the inhibitory antibodies specifically target the 5 mutated amino acids, we used an excess of biotinylated recombinant human F8 (1.2 μ g of Bio-F8) to saturate the inhibitory antibodies against regular FVIII in rat plasma. Then 30 μ l of streptavidin agarose was used to pull down antigen-antibody complexes by rotation at room

temperature for 1 hour and then centrifuged at 10,000 rpm for 2 min. An equivalent amount of biotinylated BSA (Bio-BSA) was used as a control. 200 ng of BDD-F8-X5 concentrate from a stably expressing cell line was then added to the rat plasma pretreated with Bio-F8 or Bio-BSA. Rat plasma without pretreatment was used as control. The F8 activity in the supernatant was determined by one-stage aPTT assay. As shown in FIG. 15, neutralizing antibodies against the five mutated amino acids were not detected in this F8-/- rat model.

[0181] The above Examples show that the mutant factor VIII products of the present invention express and/or secrete better than wild type factor VIII, therefore they can decrease the production cost and improve transgene expression levels

when using a gene transfer vector. They can also allow lower vector doses to be administered and higher factor VIII expression levels.

[0182] The above description is for the purpose of teaching a person of ordinary skill in the art how to practice the present invention. It is not intended to detail all those obvious modifications and variations of it which will become apparent to the skilled worker upon reading the description. It is intended, however, that all such obvious modifications and variations be included within the scope of the present invention, which is defined by the following claims. The claims are intended to cover the claimed components and steps in any sequence effective to meet the objectives there intended, unless the context specifically indicates the contrary.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 9

<210> SEQ ID NO 1

<211> LENGTH: 7056

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 1

```

atgcaaatag agctctccac ctgcttcttt ctgtgccttt tgcgattctg ctttagtgcc      60
accagaagat actacctggg tgcagtggaa ctgtcatggg actatatgca aagtgatctc      120
ggtagagctgc ctgtggacgc aagatttcct cctagagtgc caaaatcttt tccattcaac      180
acctcagtcg tgtacaaaaa gactctgttt gtagaattca cggatcacct tttcaacatc      240
gctaagccaa ggccaccctg gatgggtctg ctagggtcta ccatccaggc tgaggtttat      300
gatacagtgg tcattacact taagaacatg gcttcccatc ctgtcagtc tcatgctggt      360
gggtgatcct actggaaagc ttctgagga gctgaatatg atgatcagac cagtcaaagg      420
gagaaagaag atgataaagt cttccctggt ggaagccata catatgtctg gcaggtcctg      480
aaagagaatg gtccaatggc ctctgaccca ctgtgcctta cctactcata tctttctcat      540
gtggacctgg taaaagactt gaattcaggc ctcatggag ccctactagt atgtagagaa      600
gggagtctgg ccaaggaaaa gacacagacc ttgcacaaat ttatactact ttttctgtga      660
tttgatgaag ggaaaagtgt gcactcagaa acaaagaact ccttgatgca ggatagggat      720
gctgcatctg ctcgggcctg gcctaaaatg cacacagtca atggttatgt aaacaggtct      780
ctgccaggtc tgattggatg ccacaggaaa tcagtctatt ggcattgtgat tggaatgggc      840
accactcctg aagtgcactc aatattcctc gaaggtcaca catttcttgt gaggaacct      900
cgccaggcgt ccttggaat ctcgccaata actttcotta ctgctcaaac actcttgatg      960
gaccttgga cagtttctact gttttgtcat atctcttccc accaactatga tggcatggaa      1020
gcttatgtca aagtagacag ctgtccagag gaacccaac tacgaatgaa aaataatgaa      1080
gaagcggaag actatgatga tgatcttact gattctgaaa tggatgtggt caggtttgat      1140
gatgacaact ctccttcctt tatccaaatt cgctcagttg ccaagaagca tcctaaaact      1200
tgggtacatt acattgctgc tgaagaggag gactgggact atgctccctt agtcctcgcc      1260
cccgatgaca gaagttataa aagtcaatat ttgaacaatg gccctcagcg gattggtagg      1320
aagtacaaaa aagtcgatt tatggcatc acagatgaaa cctttaagac tcgtgaagct      1380
attcagcatg aatcaggaat cttgggacct ttactttatg gggaagttgg agacacactg      1440

```

-continued

ttgattatat	ttaagaatca	agcaagcaga	ccatataaca	tctaccctca	cggaatcact	1500
gatgtccgctc	ctttgtattc	aaggagatta	ccaaaagggtg	taaaacattt	gaaggatttt	1560
ccaattctgc	caggagaaat	attcaaatat	aatggacag	tgactgtaga	agatgggcca	1620
actaaatcag	atcctcgggtg	cctgaccgcg	tattactcta	gtttcgtaa	tatggagaga	1680
gatctagctt	caggactcat	tggccctctc	ctcatctgct	acaagaatc	tgtagatcaa	1740
agaggaaacc	agataatgtc	agacaagagg	aatgtcatcc	tgttttctgt	atttgatgag	1800
aaccgaagct	ggtacctcac	agagaatata	caacgctttc	tcccaatcc	agctggagt	1860
cagcttgagg	atccagagtt	ccaagcctcc	aacatcatgc	acagcatcaa	tggtctatgt	1920
tttgatagtt	tcagttgtc	agtttgtttg	catgaggtgg	catactggta	cattctaagc	1980
attggagcac	agactgactt	cctttctgtc	ttcttctctg	gatatacctt	caaacacaaa	2040
atggtctatg	aagacacact	caccctattc	ccattctcag	gagaaactgt	cttcatgtcg	2100
atggaaaacc	caggtctatg	gattctgggg	tgccacaact	cagactttcg	gaacagaggc	2160
atgaccgcct	tactgaaggt	ttctagtgtg	gacaagaaca	ctggtgatta	ttacgaggac	2220
agttatgaag	atatttcagc	atacttgctg	agtaaaaaca	atgccattga	accaagaagc	2280
ttctcccaga	attcaagaca	ccgtagcact	aggcaaaagc	aatttaatgc	caccacaatt	2340
ccagaaaatg	acatagagaa	gactgaccct	tggtttgcac	acagaacacc	tatgcctaaa	2400
atacaaaatg	tctcctctag	tgatttgttg	atgctcttgc	gacagagtcc	tactccacat	2460
gggtatcct	tatctgatct	ccaagaagcc	aaatatgaga	ctttttctga	tgatccatca	2520
cctggagcaa	tagacagtaa	taacagcctg	tctgaaatga	cacacttcag	gccacagctc	2580
catcacagtg	gggacatggt	atttaccctc	gagtcaggcc	tccaattaag	attaaatgag	2640
aaactgggga	caactgcagc	aacagagtgt	aagaaacttg	atttcaaagt	ttctagtaca	2700
tcaaataatc	tgatttcaac	aattccatca	gacaatttgg	cagcaggtac	tgataataca	2760
agttccttag	gaccccccaag	tatgccagtt	cattatgata	gtcaattaga	taccactcta	2820
tttggaacaa	agtcctctcc	cottactgag	tctggtggac	ctctgagctt	gagtgaagaa	2880
aataatgatt	caaagtgtgt	agaatcaggt	ttaatgaata	gccaagaaag	ttcatgggga	2940
aaaaatgtat	cgtcaacaga	gagtggtagg	ttattttaaag	ggaaaagagc	tcatggacct	3000
gctttgttga	ctaaagataa	tgcccttattc	aaagttagca	tctctttgtt	aaagacaaac	3060
aaaacttcca	ataattcagc	aactaataga	aagactcaca	ttgatggccc	atcattatta	3120
attgagaata	gtccatcagt	ctggcaaaat	atattagaaa	gtgacactga	gtttaaaaaa	3180
gtgacacctt	tgattcatga	cagaatgctt	atggacaaaa	atgetacagc	tttgaggcta	3240
aatcatatgt	caaataaaac	tacttcatca	aaaaacatgg	aatgggtcca	acagaaaaaa	3300
gaggggcccca	ttccaccaga	tgacacaaaat	ccagatatgt	cgttctttaa	gatgctattc	3360
ttgccagaat	cagcaagggtg	gatacaaaag	actcatggaa	agaactctct	gaactctggg	3420
caaggcccca	gtccaaagca	attagtatcc	ttaggaccag	aaaaatctgt	ggaagggtcag	3480
aatttcttgt	ctgagaaaaa	caaagtggta	gtaggaaagg	gtgaatttac	aaaggacgta	3540
ggactcaaaag	agatggtttt	tccaagcagc	agaaacctat	ttcttactaa	cttgataat	3600
ttacatgaaa	ataatacaca	caatcaagaa	aaaaaaattc	aggaagaaat	agaaaagaag	3660
gaaacattaa	tccaagagaa	tgtagttttg	cctcagatac	atacagtgac	tggcactaag	3720

-continued

aatttcacga	agaacctttt	cttactgagc	actaggcaaa	atgtagaagg	ttcatatgac	3780
ggggcatatg	ctccagtact	tcaagatttt	aggtcattaa	atgattcaac	aaatagaaca	3840
aagaaacaca	cagctcattt	ctcaaaaaaa	ggggaggaag	aaaacttgga	aggcttggga	3900
aatcaaacca	agcaaattgt	agagaaatat	gcacgcacca	caaggatata	tcctaataca	3960
agccagcaga	atcttctcac	gcaacgtagt	aagagagctt	tgaacaatt	cagactccca	4020
ctagaagaaa	cagaacttga	aaaaaggata	attgtggatg	acacctcaac	ccagtgggtc	4080
aaaaacatga	aacatttgac	cccagacacc	ctcacacaga	tagactacaa	tgagaaggag	4140
aaaggggcca	ttactcagtc	tcccttatca	gattgcctta	cgaggagtca	tagcatccct	4200
caagcaata	gatctccatt	accatttgca	aaggtatcat	catttccatc	tattagacct	4260
atatatctga	ccagggtcct	attccaagac	aactcttctc	atcttccagc	agcatcttat	4320
agaaagaaag	attctggggt	ccaagaaagc	agtcatttct	tacaaggagc	caaaaaaat	4380
aacctttctt	tagccattct	aaccttgag	atgactggtg	atcaaagaga	ggttggtctc	4440
ctggggacaa	gtgccacaaa	ttcagtcaca	tacaagaaag	ttgagaacac	tgttctcccg	4500
aaaccagact	tgcccaaac	atctggcaaa	gttgaaattgc	ttccaaaagt	tcacatttat	4560
cagaaggacc	tattccctac	ggaaactagc	aatgggtctc	ctggccatct	ggatctcgtg	4620
gaagggagcc	ttcttcaggg	aacagaggga	gcgattaagt	ggaatgaagc	aaacagacct	4680
ggaaaagtct	cctttctgag	agtagcaaca	gaaagctctg	caaagactcc	ctccaagcta	4740
ttggatcctc	ttgcttggga	taaccactat	ggtactcaga	tacaaaaga	agagtggaaa	4800
tcccaagaga	agtcaccaga	aaaaacagct	tttaagaaaa	aggataccat	tttgtccctg	4860
aacgcttggt	aaagcaatca	tgcaatagca	gcaataaatg	agggacaaaa	taagcccga	4920
atagaagtca	cctgggcaaa	gcaaggtagg	actgaaaggc	tgtgctctca	aaaccaccca	4980
gtcttgaaac	gccatcaacg	ggaaataact	cgtactactc	ttcagtcaga	tcaagaggaa	5040
attgactatg	atgataccat	atcagttgaa	atgaagaagg	aagattttga	catttatgat	5100
gaggatgaaa	atcagagccc	cgcagctttt	caaaagaaaa	cacgacacta	ttttattgct	5160
gcagtgagga	ggctctggga	ttatgggatg	agtagctccc	cacatgttct	aagaacaggg	5220
gctcagagtg	gcagtgctcc	tcagttcaag	aaagttgttt	tccaggaatt	tactgatggc	5280
tcctttactc	agcccttata	cgttgagaaa	ctaaatgaac	atttgggact	cctggggcca	5340
tatataagag	cagaagttag	agataatatc	atggtaactt	tcagaaatca	ggcctctcgt	5400
ccctattcct	tctattctag	ccttatttct	tataggaag	atcagaggca	aggagcagaa	5460
cctagaaaaa	actttgtcaa	gcctaatgaa	acaaaaactt	acttttggaa	agtgaacat	5520
catatggcac	ccactaaaga	tgagtttgac	tgcaaaagcct	gggcttattt	ctctgatgtt	5580
gacctggaaa	aagatgtgca	ctcaggcctg	attggacccc	ttctgggtctg	ccacactaac	5640
acactgaacc	ctgctcatgg	gagacaagtg	acagtacagg	aatttgctct	gtttttcacc	5700
atctttgatg	agacccaaa	ctggtacttc	actgaaaata	tggaaagaaa	ctgcagggct	5760
ccctgaata	tccagatgga	agatccact	tttaagaga	attatcgctt	ccatgcaatc	5820
aatggctaca	taatggatac	actacctggc	ttagtaatgg	ctcaggatca	aaggattcga	5880
tggtatctgc	tcagcatggg	cagcaatgaa	aacatccatt	ctattcattt	cagtggacat	5940
gtgttcactg	tacgaaaaaa	agaggagtat	aaaatggcac	tgtacaatct	ctatccaggt	6000

-continued

```

gtttttgaga cagtggaaat gttaccatcc aaagctggaa ttggcggggt ggaatgcctt 6060
attggcgagc atctacatgc tgggatgagc acactttttc tgggtgtacag caataagtgt 6120
cagactcccc tgggaatggc ttctggacac attagagatt ttcagattac agcttcagga 6180
caatatggac agtgggcccc aaagctggcc agacttcatt attccggatc aatcaatgcc 6240
tggagcacca aggagccctt ttctggatc aaggtggatc tgttggcacc aatgattatt 6300
cacggcatca agaccagggt tgcccgtagc aagttctcca gcctctacat ctctcagttt 6360
atcatcatgt atagtcttga tgggaagaag tggcagactt atcgaggaaa ttccactgga 6420
accttaatgg tcttcttttg caatgtggat tcactctggga taaaacacaa tatttttaac 6480
ctccaatta ttgctcgata catccgtttg cacccaactc attatagcat tcgcagcact 6540
cttcgcatgg agttgatggg ctgtgattta aatagttgca gcatgccatt gggaatggag 6600
agtaaagcaa tatcagatgc acagattact gcttcatcct actttaccaa tatgtttgcc 6660
acctggtctc cttcaaaagc tcgacttcac ctccaaggga ggagtaatgc ctggagacct 6720
caggtgaata atccaaaaga gtggctgcaa gtggacttcc agaagacaat gaaagtcaca 6780
ggagtaacta ctcaggaggt aaaatctctg cttaccagca tgtatgtgaa ggagttcctc 6840
atctccagca gtcaagatgg ccatcagtgg actctctttt ttcagaatgg caaagtaaag 6900
gtttttcagg gaaatcaaga ctccttcaca cctgtggtga actctctaga cccaccgtta 6960
ctgactcgct accttcgaat tcacccccag agttgggtgc accagattgc cctgaggatg 7020
gaggttctgg gctgcgaggc acaggacctc tactga 7056

```

```

<210> SEQ ID NO 2
<211> LENGTH: 2332
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

```

```

<400> SEQUENCE: 2

```

```

Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser Trp Asp Tyr
1      5      10      15
Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg Phe Pro Pro
20     25     30
Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val Tyr Lys Lys
35     40     45
Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile Ala Lys Pro
50     55     60
Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val
65     70     75     80
Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser His Pro Val
85     90     95
Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala
100    105    110
Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys Val
115    120    125
Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu Lys Glu Asn
130    135    140
Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu Ser
145    150    155    160
His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala Leu
165    170    175

```

-continued

Leu	Val	Cys	Arg	Glu	Gly	Ser	Leu	Ala	Lys	Glu	Lys	Thr	Gln	Thr	Leu	180	185	190
His	Lys	Phe	Ile	Leu	Leu	Phe	Ala	Val	Phe	Asp	Glu	Gly	Lys	Ser	Trp	195	200	205
His	Ser	Glu	Thr	Lys	Asn	Ser	Leu	Met	Gln	Asp	Arg	Asp	Ala	Ala	Ser	210	215	220
Ala	Arg	Ala	Trp	Pro	Lys	Met	His	Thr	Val	Asn	Gly	Tyr	Val	Asn	Arg	225	230	235
Ser	Leu	Pro	Gly	Leu	Ile	Gly	Cys	His	Arg	Lys	Ser	Val	Tyr	Trp	His	245	250	255
Val	Ile	Gly	Met	Gly	Thr	Thr	Pro	Glu	Val	His	Ser	Ile	Phe	Leu	Glu	260	265	270
Gly	His	Thr	Phe	Leu	Val	Arg	Asn	His	Arg	Gln	Ala	Ser	Leu	Glu	Ile	275	280	285
Ser	Pro	Ile	Thr	Phe	Leu	Thr	Ala	Gln	Thr	Leu	Leu	Met	Asp	Leu	Gly	290	295	300
Gln	Phe	Leu	Leu	Phe	Cys	His	Ile	Ser	Ser	His	Gln	His	Asp	Gly	Met	305	310	315
Glu	Ala	Tyr	Val	Lys	Val	Asp	Ser	Cys	Pro	Glu	Glu	Pro	Gln	Leu	Arg	325	330	335
Met	Lys	Asn	Asn	Glu	Glu	Ala	Glu	Asp	Tyr	Asp	Asp	Asp	Leu	Thr	Asp	340	345	350
Ser	Glu	Met	Asp	Val	Val	Arg	Phe	Asp	Asp	Asp	Asn	Ser	Pro	Ser	Phe	355	360	365
Ile	Gln	Ile	Arg	Ser	Val	Ala	Lys	Lys	His	Pro	Lys	Thr	Trp	Val	His	370	375	380
Tyr	Ile	Ala	Ala	Glu	Glu	Glu	Asp	Trp	Asp	Tyr	Ala	Pro	Leu	Val	Leu	385	390	395
Ala	Pro	Asp	Asp	Arg	Ser	Tyr	Lys	Ser	Gln	Tyr	Leu	Asn	Asn	Gly	Pro	405	410	415
Gln	Arg	Ile	Gly	Arg	Lys	Tyr	Lys	Lys	Val	Arg	Phe	Met	Ala	Tyr	Thr	420	425	430
Asp	Glu	Thr	Phe	Lys	Thr	Arg	Glu	Ala	Ile	Gln	His	Glu	Ser	Gly	Ile	435	440	445
Leu	Gly	Pro	Leu	Leu	Tyr	Gly	Glu	Val	Gly	Asp	Thr	Leu	Leu	Ile	Ile	450	455	460
Phe	Lys	Asn	Gln	Ala	Ser	Arg	Pro	Tyr	Asn	Ile	Tyr	Pro	His	Gly	Ile	465	470	475
Thr	Asp	Val	Arg	Pro	Leu	Tyr	Ser	Arg	Arg	Leu	Pro	Lys	Gly	Val	Lys	485	490	495
His	Leu	Lys	Asp	Phe	Pro	Ile	Leu	Pro	Gly	Glu	Ile	Phe	Lys	Tyr	Lys	500	505	510
Trp	Thr	Val	Thr	Val	Glu	Asp	Gly	Pro	Thr	Lys	Ser	Asp	Pro	Arg	Cys	515	520	525
Leu	Thr	Arg	Tyr	Tyr	Ser	Ser	Phe	Val	Asn	Met	Glu	Arg	Asp	Leu	Ala	530	535	540
Ser	Gly	Leu	Ile	Gly	Pro	Leu	Leu	Ile	Cys	Tyr	Lys	Glu	Ser	Val	Asp	545	550	555
Gln	Arg	Gly	Asn	Gln	Ile	Met	Ser	Asp	Lys	Arg	Asn	Val	Ile	Leu	Phe	565	570	575

-continued

Ser	Val	Phe	Asp	Glu	Asn	Arg	Ser	Trp	Tyr	Leu	Thr	Glu	Asn	Ile	Gln	580	585	590
Arg	Phe	Leu	Pro	Asn	Pro	Ala	Gly	Val	Gln	Leu	Glu	Asp	Pro	Glu	Phe	595	600	605
Gln	Ala	Ser	Asn	Ile	Met	His	Ser	Ile	Asn	Gly	Tyr	Val	Phe	Asp	Ser	610	615	620
Leu	Gln	Leu	Ser	Val	Cys	Leu	His	Glu	Val	Ala	Tyr	Trp	Tyr	Ile	Leu	625	630	635
Ser	Ile	Gly	Ala	Gln	Thr	Asp	Phe	Leu	Ser	Val	Phe	Phe	Ser	Gly	Tyr	645	650	655
Thr	Phe	Lys	His	Lys	Met	Val	Tyr	Glu	Asp	Thr	Leu	Thr	Leu	Phe	Pro	660	665	670
Phe	Ser	Gly	Glu	Thr	Val	Phe	Met	Ser	Met	Glu	Asn	Pro	Gly	Leu	Trp	675	680	685
Ile	Leu	Gly	Cys	His	Asn	Ser	Asp	Phe	Arg	Asn	Arg	Gly	Met	Thr	Ala	690	695	700
Leu	Leu	Lys	Val	Ser	Ser	Cys	Asp	Lys	Asn	Thr	Gly	Asp	Tyr	Tyr	Glu	705	710	715
Asp	Ser	Tyr	Glu	Asp	Ile	Ser	Ala	Tyr	Leu	Leu	Ser	Lys	Asn	Asn	Ala	725	730	735
Ile	Glu	Pro	Arg	Ser	Phe	Ser	Gln	Asn	Ser	Arg	His	Pro	Ser	Thr	Arg	740	745	750
Gln	Lys	Gln	Phe	Asn	Ala	Thr	Thr	Ile	Pro	Glu	Asn	Asp	Ile	Glu	Lys	755	760	765
Thr	Asp	Pro	Trp	Phe	Ala	His	Arg	Thr	Pro	Met	Pro	Lys	Ile	Gln	Asn	770	775	780
Val	Ser	Ser	Ser	Asp	Leu	Leu	Met	Leu	Leu	Arg	Gln	Ser	Pro	Thr	Pro	785	790	795
His	Gly	Leu	Ser	Leu	Ser	Asp	Leu	Gln	Glu	Ala	Lys	Tyr	Glu	Thr	Phe	805	810	815
Ser	Asp	Asp	Pro	Ser	Pro	Gly	Ala	Ile	Asp	Ser	Asn	Asn	Ser	Leu	Ser	820	825	830
Glu	Met	Thr	His	Phe	Arg	Pro	Gln	Leu	His	His	Ser	Gly	Asp	Met	Val	835	840	845
Phe	Thr	Pro	Glu	Ser	Gly	Leu	Gln	Leu	Arg	Leu	Asn	Glu	Lys	Leu	Gly	850	855	860
Thr	Thr	Ala	Ala	Thr	Glu	Leu	Lys	Lys	Leu	Asp	Phe	Lys	Val	Ser	Ser	865	870	875
Thr	Ser	Asn	Asn	Leu	Ile	Ser	Thr	Ile	Pro	Ser	Asp	Asn	Leu	Ala	Ala	885	890	895
Gly	Thr	Asp	Asn	Thr	Ser	Ser	Leu	Gly	Pro	Pro	Ser	Met	Pro	Val	His	900	905	910
Tyr	Asp	Ser	Gln	Leu	Asp	Thr	Thr	Leu	Phe	Gly	Lys	Lys	Ser	Ser	Pro	915	920	925
Leu	Thr	Glu	Ser	Gly	Gly	Pro	Leu	Ser	Leu	Ser	Glu	Glu	Asn	Asn	Asp	930	935	940
Ser	Lys	Leu	Leu	Glu	Ser	Gly	Leu	Met	Asn	Ser	Gln	Glu	Ser	Ser	Trp	945	950	955
Gly	Lys	Asn	Val	Ser	Ser	Thr	Glu	Ser	Gly	Arg	Leu	Phe	Lys	Gly	Lys	965	970	975
Arg	Ala	His	Gly	Pro	Ala	Leu	Leu	Thr	Lys	Asp	Asn	Ala	Leu	Phe	Lys			

-continued

980						985						990					
Val	Ser	Ile	Ser	Leu	Leu	Lys	Thr	Asn	Lys	Thr	Ser	Asn	Asn	Ser	Ala		
995						1000						1005					
Thr	Asn	Arg	Lys	Thr	His	Ile	Asp	Gly	Pro	Ser	Leu	Leu	Ile	Glu			
1010						1015						1020					
Asn	Ser	Pro	Ser	Val	Trp	Gln	Asn	Ile	Leu	Glu	Ser	Asp	Thr	Glu			
1025						1030						1035					
Phe	Lys	Lys	Val	Thr	Pro	Leu	Ile	His	Asp	Arg	Met	Leu	Met	Asp			
1040						1045						1050					
Lys	Asn	Ala	Thr	Ala	Leu	Arg	Leu	Asn	His	Met	Ser	Asn	Lys	Thr			
1055						1060						1065					
Thr	Ser	Ser	Lys	Asn	Met	Glu	Met	Val	Gln	Gln	Lys	Lys	Glu	Gly			
1070						1075						1080					
Pro	Ile	Pro	Pro	Asp	Ala	Gln	Asn	Pro	Asp	Met	Ser	Phe	Phe	Lys			
1085						1090						1095					
Met	Leu	Phe	Leu	Pro	Glu	Ser	Ala	Arg	Trp	Ile	Gln	Arg	Thr	His			
1100						1105						1110					
Gly	Lys	Asn	Ser	Leu	Asn	Ser	Gly	Gln	Gly	Pro	Ser	Pro	Lys	Gln			
1115						1120						1125					
Leu	Val	Ser	Leu	Gly	Pro	Glu	Lys	Ser	Val	Glu	Gly	Gln	Asn	Phe			
1130						1135						1140					
Leu	Ser	Glu	Lys	Asn	Lys	Val	Val	Val	Gly	Lys	Gly	Glu	Phe	Thr			
1145						1150						1155					
Lys	Asp	Val	Gly	Leu	Lys	Glu	Met	Val	Phe	Pro	Ser	Ser	Arg	Asn			
1160						1165						1170					
Leu	Phe	Leu	Thr	Asn	Leu	Asp	Asn	Leu	His	Glu	Asn	Asn	Thr	His			
1175						1180						1185					
Asn	Gln	Glu	Lys	Lys	Ile	Gln	Glu	Glu	Ile	Glu	Lys	Lys	Glu	Thr			
1190						1195						1200					
Leu	Ile	Gln	Glu	Asn	Val	Val	Leu	Pro	Gln	Ile	His	Thr	Val	Thr			
1205						1210						1215					
Gly	Thr	Lys	Asn	Phe	Met	Lys	Asn	Leu	Phe	Leu	Leu	Ser	Thr	Arg			
1220						1225						1230					
Gln	Asn	Val	Glu	Gly	Ser	Tyr	Asp	Gly	Ala	Tyr	Ala	Pro	Val	Leu			
1235						1240						1245					
Gln	Asp	Phe	Arg	Ser	Leu	Asn	Asp	Ser	Thr	Asn	Arg	Thr	Lys	Lys			
1250						1255						1260					
His	Thr	Ala	His	Phe	Ser	Lys	Lys	Gly	Glu	Glu	Glu	Asn	Leu	Glu			
1265						1270						1275					
Gly	Leu	Gly	Asn	Gln	Thr	Lys	Gln	Ile	Val	Glu	Lys	Tyr	Ala	Cys			
1280						1285						1290					
Thr	Thr	Arg	Ile	Ser	Pro	Asn	Thr	Ser	Gln	Gln	Asn	Phe	Val	Thr			
1295						1300						1305					
Gln	Arg	Ser	Lys	Arg	Ala	Leu	Lys	Gln	Phe	Arg	Leu	Pro	Leu	Glu			
1310						1315						1320					
Glu	Thr	Glu	Leu	Glu	Lys	Arg	Ile	Ile	Val	Asp	Asp	Thr	Ser	Thr			
1325						1330						1335					
Gln	Trp	Ser	Lys	Asn	Met	Lys	His	Leu	Thr	Pro	Ser	Thr	Leu	Thr			
1340						1345						1350					
Gln	Ile	Asp	Tyr	Asn	Glu	Lys	Glu	Lys	Gly	Ala	Ile	Thr	Gln	Ser			
1355						1360						1365					

-continued

Pro Leu	Ser Asp Cys Leu Thr	Arg Ser His Ser Ile	Pro Gln Ala
1370	1375	1380	
Asn Arg	Ser Pro Leu Pro Ile	Ala Lys Val Ser Ser	Phe Pro Ser
1385	1390	1395	
Ile Arg	Pro Ile Tyr Leu Thr	Arg Val Leu Phe Gln	Asp Asn Ser
1400	1405	1410	
Ser His	Leu Pro Ala Ala Ser	Tyr Arg Lys Lys Asp	Ser Gly Val
1415	1420	1425	
Gln Glu	Ser Ser His Phe Leu	Gln Gly Ala Lys Lys	Asn Asn Leu
1430	1435	1440	
Ser Leu	Ala Ile Leu Thr Leu	Glu Met Thr Gly Asp	Gln Arg Glu
1445	1450	1455	
Val Gly	Ser Leu Gly Thr Ser	Ala Thr Asn Ser Val	Thr Tyr Lys
1460	1465	1470	
Lys Val	Glu Asn Thr Val Leu	Pro Lys Pro Asp Leu	Pro Lys Thr
1475	1480	1485	
Ser Gly	Lys Val Glu Leu Leu	Pro Lys Val His Ile	Tyr Gln Lys
1490	1495	1500	
Asp Leu	Phe Pro Thr Glu Thr	Ser Asn Gly Ser Pro	Gly His Leu
1505	1510	1515	
Asp Leu	Val Glu Gly Ser Leu	Leu Gln Gly Thr Glu	Gly Ala Ile
1520	1525	1530	
Lys Trp	Asn Glu Ala Asn Arg	Pro Gly Lys Val Pro	Phe Leu Arg
1535	1540	1545	
Val Ala	Thr Glu Ser Ser Ala	Lys Thr Pro Ser Lys	Leu Leu Asp
1550	1555	1560	
Pro Leu	Ala Trp Asp Asn His	Tyr Gly Thr Gln Ile	Pro Lys Glu
1565	1570	1575	
Glu Trp	Lys Ser Gln Glu Lys	Ser Pro Glu Lys Thr	Ala Phe Lys
1580	1585	1590	
Lys Lys	Asp Thr Ile Leu Ser	Leu Asn Ala Cys Glu	Ser Asn His
1595	1600	1605	
Ala Ile	Ala Ala Ile Asn Glu	Gly Gln Asn Lys Pro	Glu Ile Glu
1610	1615	1620	
Val Thr	Trp Ala Lys Gln Gly	Arg Thr Glu Arg Leu	Cys Ser Gln
1625	1630	1635	
Asn Pro	Pro Val Leu Lys Arg	His Gln Arg Glu Ile	Thr Arg Thr
1640	1645	1650	
Thr Leu	Gln Ser Asp Gln Glu	Glu Ile Asp Tyr Asp	Asp Thr Ile
1655	1660	1665	
Ser Val	Glu Met Lys Lys Glu	Asp Phe Asp Ile Tyr	Asp Glu Asp
1670	1675	1680	
Glu Asn	Gln Ser Pro Arg Ser	Phe Gln Lys Lys Thr	Arg His Tyr
1685	1690	1695	
Phe Ile	Ala Ala Val Glu Arg	Leu Trp Asp Tyr Gly	Met Ser Ser
1700	1705	1710	
Ser Pro	His Val Leu Arg Asn	Arg Ala Gln Ser Gly	Ser Val Pro
1715	1720	1725	
Gln Phe	Lys Lys Val Val Phe	Gln Glu Phe Thr Asp	Gly Ser Phe
1730	1735	1740	

-continued

Thr 1745	Gln	Pro	Leu	Tyr	Arg	Gly 1750	Glu	Leu	Asn	Glu	His 1755	Leu	Gly	Leu
Leu 1760	Gly	Pro	Tyr	Ile	Arg	Ala 1765	Glu	Val	Glu	Asp	Asn 1770	Ile	Met	Val
Thr 1775	Phe	Arg	Asn	Gln	Ala	Ser 1780	Arg	Pro	Tyr	Ser	Phe 1785	Tyr	Ser	Ser
Leu 1790	Ile	Ser	Tyr	Glu	Glu	Asp 1795	Gln	Arg	Gln	Gly	Ala 1800	Glu	Pro	Arg
Lys 1805	Asn	Phe	Val	Lys	Pro	Asn 1810	Glu	Thr	Lys	Thr	Tyr 1815	Phe	Trp	Lys
Val 1820	Gln	His	His	Met	Ala	Pro 1825	Thr	Lys	Asp	Glu	Phe 1830	Asp	Cys	Lys
Ala 1835	Trp	Ala	Tyr	Phe	Ser	Asp 1840	Val	Asp	Leu	Glu	Lys 1845	Asp	Val	His
Ser 1850	Gly	Leu	Ile	Gly	Pro	Leu 1855	Leu	Val	Cys	His	Thr 1860	Asn	Thr	Leu
Asn 1865	Pro	Ala	His	Gly	Arg	Gln 1870	Val	Thr	Val	Gln	Glu 1875	Phe	Ala	Leu
Phe 1880	Phe	Thr	Ile	Phe	Asp	Glu 1885	Thr	Lys	Ser	Trp	Tyr 1890	Phe	Thr	Glu
Asn 1895	Met	Glu	Arg	Asn	Cys	Arg 1900	Ala	Pro	Cys	Asn	Ile 1905	Gln	Met	Glu
Asp 1910	Pro	Thr	Phe	Lys	Glu	Asn 1915	Tyr	Arg	Phe	His	Ala 1920	Ile	Asn	Gly
Tyr 1925	Ile	Met	Asp	Thr	Leu	Pro 1930	Gly	Leu	Val	Met	Ala 1935	Gln	Asp	Gln
Arg 1940	Ile	Arg	Trp	Tyr	Leu	Leu 1945	Ser	Met	Gly	Ser	Asn 1950	Glu	Asn	Ile
His 1955	Ser	Ile	His	Phe	Ser	Gly 1960	His	Val	Phe	Thr	Val 1965	Arg	Lys	Lys
Glu 1970	Glu	Tyr	Lys	Met	Ala	Leu 1975	Tyr	Asn	Leu	Tyr	Pro 1980	Gly	Val	Phe
Glu 1985	Thr	Val	Glu	Met	Leu	Pro 1990	Ser	Lys	Ala	Gly	Ile 1995	Trp	Arg	Val
Glu 2000	Cys	Leu	Ile	Gly	Glu	His 2005	Leu	His	Ala	Gly	Met 2010	Ser	Thr	Leu
Phe 2015	Leu	Val	Tyr	Ser	Asn	Lys 2020	Cys	Gln	Thr	Pro	Leu 2025	Gly	Met	Ala
Ser 2030	Gly	His	Ile	Arg	Asp	Phe 2035	Gln	Ile	Thr	Ala	Ser 2040	Gly	Gln	Tyr
Gly 2045	Gln	Trp	Ala	Pro	Lys	Leu 2050	Ala	Arg	Leu	His	Tyr 2055	Ser	Gly	Ser
Ile 2060	Asn	Ala	Trp	Ser	Thr	Lys 2065	Glu	Pro	Phe	Ser	Trp 2070	Ile	Lys	Val
Asp 2075	Leu	Leu	Ala	Pro	Met	Ile 2080	Ile	His	Gly	Ile	Lys 2085	Thr	Gln	Gly
Ala 2090	Arg	Gln	Lys	Phe	Ser	Ser 2095	Leu	Tyr	Ile	Ser	Gln 2100	Phe	Ile	Ile
Met 2105	Tyr	Ser	Leu	Asp	Gly	Lys 2110	Lys	Trp	Gln	Thr	Tyr 2115	Arg	Gly	Asn
Ser	Thr	Gly	Thr	Leu	Met	Val	Phe	Phe	Gly	Asn	Val	Asp	Ser	Ser

-continued

2120	2125	2130
Gly Ile Lys His Asn Ile Phe Asn Pro Pro Ile Ile Ala Arg Tyr 2135 2140 2145		
Ile Arg Leu His Pro Thr His Tyr Ser Ile Arg Ser Thr Leu Arg 2150 2155 2160		
Met Glu Leu Met Gly Cys Asp Leu Asn Ser Cys Ser Met Pro Leu 2165 2170 2175		
Gly Met Glu Ser Lys Ala Ile Ser Asp Ala Gln Ile Thr Ala Ser 2180 2185 2190		
Ser Tyr Phe Thr Asn Met Phe Ala Thr Trp Ser Pro Ser Lys Ala 2195 2200 2205		
Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp Arg Pro Gln Val 2210 2215 2220		
Asn Asn Pro Lys Glu Trp Leu Gln Val Asp Phe Gln Lys Thr Met 2225 2230 2235		
Lys Val Thr Gly Val Thr Thr Gln Gly Val Lys Ser Leu Leu Thr 2240 2245 2250		
Ser Met Tyr Val Lys Glu Phe Leu Ile Ser Ser Ser Gln Asp Gly 2255 2260 2265		
His Gln Trp Thr Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe 2270 2275 2280		
Gln Gly Asn Gln Asp Ser Phe Thr Pro Val Val Asn Ser Leu Asp 2285 2290 2295		
Pro Pro Leu Leu Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp 2300 2305 2310		
Val His Gln Ile Ala Leu Arg Met Glu Val Leu Gly Cys Glu Ala 2315 2320 2325		
Gln Asp Leu Tyr 2330		

<210> SEQ ID NO 3
 <211> LENGTH: 9720
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: pAAV-CB-hBDD-F8-X10

<400> SEQUENCE: 3

```

aattcccatc atcaataata taccttattt tggattgaag ccaatatgat aatgaggggg      60
tggagtttgt gacgtggcgc ggggcggtggg aacggggcgg gtagcgtagt agtctctaga      120
ggtccccagc gaccttgacg ggcattctgcc cggcatttct gacagctttg tgaactgggt      180
ggccgagaag gaatgggagt tgccgccaga ttctgacatg gatctgaatc tgattgagca      240
ggcaccctgt accgtggcgc agaagctgca tcgctggcgt aatagcgaag aggcccgcac      300
cgatcgccct tcccaacagt tgcgcagcct gaatggcgaa tggaattcca gacgattgag      360
cgtcaaaatg taggtatttc catgagcgtt tttcctgttg caatggctgg cggtaatatt      420
gtttctggata ttaccagcaa ggccgatagt ttgagttctt ctactcaggc aagtgatgtt      480
attactaatc aaagaagtat tgcgacaacg gttaatttgc gtgatggaca gactctttta      540
ctcggtggcc tcaactgatta taaaaacact totcaggatt ctggcgtagc gttcctgtct      600
aaaaatccct taatcggcct cctgttttagc tcccgctctg attctaacga ggaaagcacg      660

```

-continued

ttatacgtgc	tcgtcaaagc	aaccatagta	cgcgccctgt	agcggcgcat	taagcgcggc	720
gggtgtgggtg	gttacgcgca	gcgtgaccgc	tacacttgcc	agcgcctag	cgcccgtcc	780
tttcgcttct	ttcccttct	ttctcgccac	gttcgcggc	tttcccgtc	aagctctaaa	840
tcgggggctc	ccttttaggt	tccgatttag	tgctttacgg	cacctcgacc	ccaaaaaact	900
tgattaggg	gatggttcac	gtagtggg	atcgccctga	tagacggttt	ttcgcccttt	960
gacgttgga	ttcacgttct	ttaatagtgg	actcttggtc	caaactggaa	caacactcaa	1020
ccctatctcg	gtctattctt	ttgatttata	agggattttg	ccgatttcgg	cctattgggt	1080
aaaaaatgag	ctgatttaac	aaaaatttaa	cgcgaatttt	aacaaaatat	taacgtttac	1140
aatttaata	tttgcttata	caatcttct	gtttttggg	cttttctgat	tatcaaccgg	1200
ggtacatatg	attgacatgc	tagttttacg	attaccgttc	atcgccctga	gggggggggg	1260
gggggggggt	ggccactccc	tctctgcgcg	ctcgtctgct	cactgaggcc	gggcgaccaa	1320
aggtcgcccc	acgccggggc	tttgccggg	cgccctcagt	gagcgagcga	gcgcgcagag	1380
agggagtggc	caactccatc	actaggggtt	cctagatctg	aattcggtag	gtacctctgg	1440
tcgttacata	acttacggta	aatggcccg	ctggctgacc	gccaacgac	cccgccatt	1500
gacgtcaata	atgacgtatg	ttcccatagt	aacgccaata	gggactttcc	attgacgtca	1560
atgggtggag	tattttacgt	aaactgccca	cttggcagta	catcaagtgt	atcatatgcc	1620
aagtacgccc	cctattgacg	tcaatgacgg	taaatggccc	gcctggcatt	atgccagta	1680
catgacctta	tgggactttc	ctacttggca	gtacatctac	tcgaggccac	gttctgcttc	1740
actctcccca	tctccccccc	ctccccccc	ccaattttgt	atttatttat	tttttaatta	1800
ttttgtgcag	cgatgggggc	gggggggggg	gggggggggg	cgcgcgccag	gcggggcggg	1860
gcggggcgag	ggggggggcg	gggcgaggcg	gagaggtgcg	gcggcagcca	atcagagcgg	1920
cgcgctccga	aagtttcctt	ttatggcgag	gcggcgggcg	cggcggccct	ataaaaagcg	1980
aagcgcgcgg	cgggcgggag	cgggatcagc	caccgcggtg	gcggccctaga	gtcgacgagg	2040
aactgaaaaa	ccagaaagtt	aactggtaag	tttagtcttt	ttgtctttta	tttcagggtc	2100
cggatccggt	ggtggtgcaa	atcaaagaac	tgctcctcag	tggatgttgc	ctttacttct	2160
aggcctgtac	ggaagtgtta	cttctgctct	aaaagctgcg	gaattgtacc	cgcggccgct	2220
tttcaaaatg	caaatagagc	tctccacctg	cttctttctg	tgccttttgc	gattctgctt	2280
tagtgccacc	agaagatact	acctgggtgc	agtggaaactg	tcattgggact	atatgcaaag	2340
tgatctcggt	gagctgcctg	tggacgcaag	atttcctcct	agagtgcaca	aatcttttcc	2400
attcaacacc	tcagtcgtgt	acaaaaagac	tctgtttgta	gaattcacgg	atcacctttt	2460
caacatcgct	aagccaaggc	caccctggat	gggtctgcta	ggtcctacca	tccaggctga	2520
ggttttacgac	acgggtggtc	ttaccctgaa	gaacatggct	tctcatcccg	ttagtcttca	2580
cgtgtcggc	gtctccttct	ggaaatcttc	cgaaggcgct	gaatatgagg	atcacaccag	2640
ccaaaggagg	aaggaagacg	ataaagtcct	tcccggtaaa	agccaaacct	acgtctggca	2700
ggtcctgaaa	gaaaatggtc	caacagcctc	tgaccaccca	tgtcttacct	actcatacct	2760
gtctcacgtg	gacctgggtg	aagacctgaa	ttcgggcctc	attggagccc	tactagtatg	2820
tagagaagg	agtctggcca	aggaaaagac	acagaccttg	cacaaattta	tactactttt	2880
tgctgtattt	gatgaaggga	aaagtggca	ctcagaaaca	aagaactcct	tgatgcagga	2940

-continued

tagggatgct	gcactctgctc	gggcctggcc	taaaatgcac	acagtcaatg	gttatgtaaa	3000
cagggtctctg	ccagggtctga	ttggatgcc	caggaaatca	gtctattggc	atgtgattgg	3060
aatgggcacc	actcctgaag	tgactcaat	attcctcgaa	ggtcacacat	ttcttgtgag	3120
gaaccatcgc	caggcgtcct	tggaaatctc	gccataaact	ttccttactg	ctcaaacact	3180
cttgatggac	cttgacagct	ttctactgtt	ttgtcatatc	tcttcccacc	aacatgatgg	3240
catggaagct	tatgtcaaag	tagacagctg	tccagaggaa	ccccaaactac	gaatgaaaaa	3300
taatgaagaa	gcggaagact	atgatgatga	tcttactgat	tctgaaatgg	atgtggtcag	3360
gtttgatgat	gacaactctc	cttcctttat	ccaaattcgc	tcagttgcc	agaagcatcc	3420
taaaacttgg	gtacattaca	ttgctgctga	agaggaggac	tgggactatg	ctcccttagt	3480
cctcgcccc	gatgacagaa	gttataaaa	tcaatatttg	aacaatggcc	ctcagcggat	3540
tggtaggaag	tacaaaaaag	tccgatttat	ggcatacaca	gatgaaacct	ttaagactcg	3600
tgaagctatt	cagcatgaat	caggaatctt	gggaccttta	ctttatgggg	aagttggaga	3660
cacactgttg	attatattta	agaatcaagc	aagcagacca	tataacatct	accctcacgg	3720
aatcactgat	gtccgtcctt	tgtattcaag	gagattacca	aaagggtgaa	aacatttgaa	3780
ggattttcca	attctgccag	gagaaatatt	caaataataa	tggacagtga	ctgtagaaga	3840
tgggccaaact	aaatcagatc	ctcgggtgcct	gacccgctat	tactctagtt	tcgttaatat	3900
ggagagagat	ctagcttcag	gactcattgg	ccctctctc	atctgctaca	aagaatctgt	3960
agatcaaaga	ggaaaccaga	taatgtcaga	caagaggaa	gtcatcctgt	tttctgtatt	4020
tgatgagaac	cgaagctggt	acctcacaga	gaatatacaa	cgctttctcc	ccaatccagc	4080
tggagtgcag	cttgaggatc	cagagttcca	agcctccaac	atcatgcaca	gcacaaatgg	4140
ctatgttttt	gatagtttgc	agttgtcagt	ttgtttgcat	gaggtggcat	actggtacat	4200
tctaagcatt	ggagcacaga	ctgacttcct	ttctgtcttc	ttctctggat	ataccttcaa	4260
acacaaaatg	gtctatgaag	acacactcac	cctattccca	ttctcaggag	aaactgtctt	4320
catgtcgtg	gaaaaccag	gtctatggat	tctgggggtgc	cacaactcag	actttcggaa	4380
cagaggcatg	accgccttac	tgaaggttct	tagttgtgac	aagaacactg	gtgattatta	4440
cgaggacagt	tatgaagata	tttcagcata	cttgctgagt	aaaaacaatg	ccattgaacc	4500
aagaagcttc	tcccagaatt	caagacaccc	tagcactagg	caaaagcaat	ttaatgccac	4560
cacaccacca	gtcttgaaac	gccatcaacg	cgaaataact	cgtactactc	ttcagtcaga	4620
tcaagaggaa	attgactatg	atgataccat	atcagttgaa	atgaagaagg	aagattttga	4680
catttatgat	gaggatgaaa	atcagagccc	ccgcagcttt	caaaagaaaa	cacgacacta	4740
ttttattgct	gcagtgagga	ggctctggga	ttatgggatg	agtagctccc	cacatgttct	4800
aagaaacagg	gtcagagtgt	gcagtgctcc	tcagttcaag	aaagttgttt	tccagggaatt	4860
tactgatggc	tcctttactc	agcccttata	cgtggagaa	ctaaatgaac	atttgggact	4920
cctggggcca	tatataagag	cagaagtga	agataaatatc	atggtaactt	tcagaaatca	4980
ggcctctcgt	ccctattcct	tctattctag	ccttatttct	tatgaggaag	atcagaggca	5040
aggagcagaa	cctagaaaaa	actttgtcaa	gcctaataaa	acaaaaactt	acttttgga	5100
agtgaacat	catatggcac	ccactaaaga	tgagtttgac	tgcaaacctt	gggcttattt	5160
ctctgatgtt	gacctggaaa	aagatgtgca	ctcaggcctg	attggacccc	ttctggctctg	5220

-continued

ccacactaac	acactgaacc	ctgctcatgg	gagacaagtg	acagtacagg	aatttgctct	5280
gtttttcacc	atctttgatg	agaccaaag	ctggacttc	actgaaaata	tggaaagaaa	5340
ctgcagggct	ccctgcaata	tccagatgga	agatcccact	tttaaagaga	attatcgctt	5400
ccatgcaatc	aatggctaca	taatggatag	actacctggc	ttagtaatgg	ctcaggatca	5460
aaggattcga	tggatatctg	tcagcatggg	cagcaatgaa	aacatccatt	ctattcattt	5520
cagtggacat	gtgttactg	tacgaaaaaa	agaggagtat	aaaatggcac	tgtacaatct	5580
ctatccaggt	gtttttgaga	cagtggaaat	gttaccatcc	aaagctggaa	tttggcgggt	5640
ggaatgcctt	attggcgagc	atctacatgc	tgggatgagc	acactttttc	tgggtgtacag	5700
caataagtg	cagactcccc	tgggaatggc	ttctggacac	attagagatt	ttcagattac	5760
agcttcagga	caatatggac	agtgggcccc	aaagctggcc	agacttcatt	attccggatc	5820
aatcaatgcc	tggagcacca	aggagccctt	ttcttggatc	aaggtggatc	tgttggcacc	5880
aatgattatt	cacggcatca	agaccaggg	tgcccgctcag	aagttctcca	gctctacat	5940
ctctcagttt	atcatcatgt	atagttctga	tgggaagaag	tggcagactt	atcgaggaaa	6000
ttccactgga	accttaattg	tcttctttgg	caatgtggat	tcactctgga	taaaacacaa	6060
tatttttaac	cctccaatta	ttgctcgata	catccgtttg	cacccaactc	attatagcat	6120
tcgcagcact	cttcgcatgg	agttgatggg	ctgtgattta	aatagttgca	gcatgccatt	6180
gggaatggag	agtaaagcaa	tatcagatgc	acagattact	gcttcaccc	actttaccaa	6240
tatgtttgcc	acctggtctc	cttcaaaagc	tcgacttcac	ctccaaggga	ggagtaatgc	6300
ctggagacct	cagggtgaata	atccaaaaga	gtggctgcaa	gtggacttcc	agaagacaat	6360
gaaagtcaca	ggagtaacta	ctcagggagt	aaaatctctg	cttaccagca	tgtatgtgaa	6420
ggagtctctc	atctccagca	gtcaagatgg	ccatcagtgg	actctctttt	ttcagaatgg	6480
caaagtaaag	gtttttcagg	gaaatcaaga	ctccttcaca	cctgtggtga	actctctaga	6540
cccaccgtta	ctgactcgct	accttcgaat	tcacccccag	agttgggtgc	accagattgc	6600
cctgaggatg	gagggttctg	gctgcgaggg	acaggacctc	tactgacaat	tgaactgtat	6660
cagcctcgac	tgtgccttct	agttgccagc	catctgttgt	tgccccctcc	cccgtgcctt	6720
ccttgaccct	ggaaggtgcc	actcccactg	tcctttccta	ataaaatgag	gaaattgcat	6780
cgcattgtct	gagtaggtgt	cattctatct	tggggggtgg	gggtggggcag	gacagcaagg	6840
gggaggattg	ggaagacaat	agcaggcatg	ctggggagag	atctaggaac	ccctagtgat	6900
ggagtgggcc	actccctctc	tgcgcgctcg	ctcgtctact	gaggccgccc	gggcaaagcc	6960
cggcgctcgg	gcgacctttg	gtcgcggggc	ctcagtgagc	gagcgagcgc	gcagagaggg	7020
agtggccaac	cccccccccc	ccccccctgc	aggcgattct	cttgtttgct	ccagactctc	7080
aggcaatgac	ctgatagcct	ttgtagagac	ctctcaaaaa	tagctaccct	ctccggcatg	7140
aatttatcag	ctagaacggt	tgaatatcat	attgatggtg	atttgactgt	ctccggcctt	7200
tctcaccctg	ttgaatcttt	acctacacat	tactcaggca	ttgcatttaa	aatatatgag	7260
ggttctaaaa	atttttatcc	ttgcgttgaa	ataaaggctt	ctcccgcaaa	agtattacag	7320
ggtcataatg	tttttggtac	aaccgattta	gctttatgct	ctgaggcttt	attgcttaat	7380
tttgctaatt	ctttgccttg	cctgtatgat	ttattggatg	ttggaattcc	tgatgcggta	7440
ttttctcctt	acgcactctg	gcggtatttc	acaccgcata	tgggtgcactc	tcagtacaat	7500

-continued

ctgctctgat gccgcatagt taagccagcc ccgacacccg ccaacacccg ctgacgcgcc	7560
ctgacgggct tgtctgctcc cggcacccg ttacagacaa gctgtgaccg tctccgggag	7620
ctgcatgtgt cagaggtttt caccgtcatc accgaaacgc gcgagacgaa agggcctcgt	7680
gatacgcccta tttttatagg ttaatgtcat gataataatg gtttcttaga cgtcaggtgg	7740
cacttttcgg ggaaatgtgc gcggaacccc tatttgttta tttttctaaa tacattcaaa	7800
tatgtatccg ctcatgagac aataaccctg ataaatgctt caataatatt gaaaaaggaa	7860
gagtatgagt attcaacatt tccgtgtcgc cettattccc tttttgccc cattttgcct	7920
tctgttttt gctcaccag aaacgctggg gaaagtaaaa gatgctgaag atcagttggg	7980
tgacgagtg gggtacatcg aactggatct caacagcggg aagatccttg agagttttcg	8040
ccccgaagaa cgttttccaa tgatgagcac ttttaaagtt ctgctatgtg gcgcgggtatt	8100
atcccgatg gacgcggggc aagagcaact cggtcgccc atacactatt ctgagaatga	8160
cttggttgag tactcaccag tcacagaaaa gcattctacg gatggcatga cagtaagaga	8220
attatgcagt gctgccataa ccatgagtga taacactgcg gccaaactac ttctgacaac	8280
gatcggagga ccgaaggagc taaccgcttt ttgcacaaac atgggggatc atgtaactcg	8340
ccttgatcgt tgggaaccgg agctgaatga agccatacca aacgacgagc gtgacaccac	8400
gatgcctgta gcaatggcaa caacgttgcg caaactatta actggcgaac tacttactct	8460
agcttcccg caacaattaa tagactggat ggaggcggat aaagtgcag gaccacttct	8520
gcgctcgcc cttccgctg gctggtttat tgctgataaa tctggagccg gtgagcgtgg	8580
gtctcgggt atcattgcag cactggggcc agatggtaag cctcccgtg tcgtagtatt	8640
ctacacgacg gggagtcagg caactatgga tgaacgaaat agacagatcg ctgagatagg	8700
tgctcactg attaagcatt ggtaactgtc agaccaagtt tactcatata tacttttagat	8760
tgatttaaaa cttcattttt aatttaaaag gatctaggtg aagatccttt ttgataatct	8820
catgacaaa atcccttaac gtgagttttc gttccactga gcgtcagacc ccgtagaaaa	8880
gatcaaaagg tcttcttgag atcctttttt tctgcgctg atctgctgct tgcaaacaaa	8940
aaaaccaccg ctaccagcgg tggtttgttt gccggatcaa gagctaccaa ctctttttcc	9000
gaaggtaact ggcttcagca gagcgcagat accaaatact gtccttctag tgtagccgta	9060
gttaggccac cacttcaaga actctgtagc accgcctaca tacctcgtc tgctaactct	9120
gttaccagt gctgctgcca gtggcgataa gtcgtgtctt accgggttg actcaagacg	9180
atagttaccg gataaggcgc agcggtcggg ctgaacgggg gggtcgtgca cacagcccag	9240
cttgagcga acgacctaca ccgaactgag atacctacag cgtgagctat gagaaagcgc	9300
cacgcttccc gaaggagaa aggcggacag gtatccgta agcggcaggg tcggaacagg	9360
agagcgcagc agggagcttc cagggggaaa cgctggtat ctttatagtc ctgtcgggtt	9420
tcgccacctc tgacttgagc gtcgattttt gtgatgctcg tcaggggggc ggagcctatg	9480
gaaaaacgcc agcaacgcgg cctttttacg gttcctggcc ttttgcggc cttttgctca	9540
catgttcttt cctgcgttat cccctgattc tgtggataac cgtattaccg cctttgagt	9600
agctgatacc gctcgcgcga gccgaacgac cgagcgcagc gagtcagtga gcgaggaagc	9660
ggaagagcgc ccaatacgca aaccgcctct cccgcgcgt tgccgattc attaatgcag	9720

-continued

<211> LENGTH: 9720
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: pAAV-CB-hBB-F8-X5

<400> SEQUENCE: 4

aattcccatc atcaataata taccttattt tggattgaag ccaatatgat aatgaggggg 60
tggagtttgt gacgtggcgc ggggcgtggg aacggggcgg gtgacgtagt agtctctaga 120
ggtecccagc gaccttgacg ggcatctgcc cggcatttct gacagctttg tgaactgggt 180
ggcgcagaag gaatgggagt tgccgccaga ttctgacatg gatctgaatc tgattgagca 240
ggcaccctct accgtggcgc agaagctgca tcgctggcgt aatagcgaag aggcccgcac 300
cgatcgccct tcccaacagt tgcgcagcct gaatggcgaa tggaaattcca gacgattgag 360
cgtcaaaatg taggtatttc catgagcgtt tttcctgttg caatggctgg cggtaatat 420
gttctggata ttaccagcaa ggccgatagt ttgagttctt ctactcaggc aagtgatgtt 480
attactaatc aaagaagtat tgcgacaacg gtttaattgc gtgatggaca gactctttta 540
ctcggtggcc tcaactgatta taaaacact tctcaggatt ctggcgtacc gttcctgtct 600
aaaaatccct taatcgccct cctgttttag tcccgtctcg attctaacga ggaaagcacg 660
ttatacgtgc tcgtcaaagc aaccatagta cgcgccctgt agcggcgcac taagcgcggc 720
gggtgtgggt gttacgcgca gcgtgaccgc tacacttgcc agcgccttag cgcgcgtcc 780
tttcgtcttc ttccttctct ttctcgccac gttcgccggc tttcccgctc aagctctaaa 840
tcgggggctc cctttagggt tccgatttag tgctttacgg cacctcgacc ccaaaaaact 900
tgattagggt gatggttcac gtagtgggcc atcgccctga tagacggttt ttcgcccttt 960
gacgttgagg tccacgttct ttaatagtgg actctgttc caaactggaa caaactcaa 1020
ccctatctcg gtctattctt ttgatttata agggattttg ccgatttcgg cctattggtt 1080
aaaaaatgag ctgatttaac aaaaatttaa cgcgaatttt aacaaaatat taacgtttac 1140
aatttaaata tttgcttata caatcttctt gtttttgggg cttttctgat tatcaaccgg 1200
ggtacatatg attgacatgc tagttttacg attaccgttc atcgccctga gggggggggg 1260
gggggggggt ggccactccc tctctgcgcg ctgcctcgct cactgaggcc gggcgaccaa 1320
aggtcgcccc acgccggggc tttgcccggg cggcctcagt gagcgagcga gcgcgcagag 1380
agggagtggc caactccatc actaggggtt cctagatctg aattcggtac gtacctctgg 1440
tcgttacata acttacggta aatggccgcg ctggctgacc gcccaacgac cccgccatt 1500
gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc attgacgtca 1560
atgggtggag tatttacggt aaactgccc cttggcagta catcaagtgt atcatatgcc 1620
aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt atgccagta 1680
catgacctta tgggactttc ctacttgcca gtacatctac tcgaggccac gttctgcttc 1740
actctcccca tctccccccc ctccccccc ccaattttgt atttatttat tttttaatta 1800
ttttgtcgag cgatgggggc gggggggggg gggggggggg cgcgcgccag gcggggcggg 1860
gcggggcgag gggcggggcg gggcgaggcg gagaggcgcg gcggcagcca atcagagcgg 1920
cgcgctccga aagtttcctt ttatggcgag gcggcgggcg cggcgccctc ataaaaagcg 1980
aagcgcgcgg cggcggggag cgggatcagc caccgcggtg gcggcctaga gtcgacgagg 2040

-continued

aactgaaaaa	ccagaaagtt	aactggtaag	tttagtcttt	ttgtctttta	tttcaggtcc	2100
cggatccggt	ggtggtgcaa	atcaaagaac	tgtcctcag	tggatgttg	ctttacttct	2160
aggcctgtac	ggaagtgtta	cttctgtct	aaaagctg	gaattgtacc	cgcggccgct	2220
tttcaaatg	caaatagagc	tctccacctg	cttctttctg	tgccttttgc	gattctgctt	2280
tagtgccacc	agaagatact	acctgggtgc	agtggaactg	tcatgggact	atatgcaaag	2340
tgatctcggt	gagctgcctg	tggacgcaag	atttctctct	agagtgcctc	aatcttttcc	2400
attcaacacc	tcagtcgtgt	acaaaaagac	tctgtttgta	gaattcacgg	atcacctttt	2460
caacatcgct	aagccaaggc	cacctggat	gggtctgcta	ggctctacca	tccaggtgta	2520
ggtttatgat	acagtgtgct	ttacacttaa	gaacatggct	tccatctctg	tcagtcttca	2580
tgctgttggt	gtatcttact	ggaaatcttc	tgaggagct	gaatatgatg	atcagaccag	2640
tcaaagggag	aaagaagatg	ataaagtctt	cctgggtaaa	agccatacat	atgtctggca	2700
ggctctgaaa	gagaatggct	caacagcttc	tgaccacca	tgccttacct	actcatatct	2760
tttcatgtg	gacctggtaa	aagacttgaa	ttcaggcttc	attggagccc	tactagtatg	2820
tagagaagg	agtctggcca	aggaaaagac	acagaccttg	cacaaattta	tactactttt	2880
tgctgtat	gatgaaggga	aaagtggcca	ctcagaaaca	aagaactcct	tgatgcagga	2940
tagggatgct	gcattctgct	gggctggcc	taaaatgcac	acagtcaatg	gttatgtaaa	3000
caggctctct	ccaggctctga	ttggatgcca	caggaaatca	gtctattggc	atgtgattgg	3060
aatgggcacc	actctgaag	tgcactcaat	attcctcgaa	ggctcacacat	ttcttgtgag	3120
gaaccatcgc	caggcgtcct	tggaaatctc	gccaataact	ttccttactg	ctcaaacact	3180
cttgatggac	cttgacagct	ttctactgtt	ttgtcatatc	tcttcccacc	aacatgatgg	3240
catggaagct	tatgtcaaa	tagacagctg	tccagaggaa	ccccaaactac	gaatgaaaaa	3300
taatgaagaa	gcggaagact	atgatgatga	tcttactgat	tctgaaatgg	atgtggtcag	3360
gtttgatgat	gacaactctc	cttcttttat	ccaaattcgc	tcagttgcca	agaagcatcc	3420
taaaacttgg	gtacattaca	ttgctgtgta	agaggaggac	tgggactatg	ctcccttagt	3480
cctcgcccc	gatgacagaa	gttataaaag	tcaatatttg	aacaatggcc	ctcagcggat	3540
tggtaggaag	tacaaaaaag	tccgatttat	ggcatacaca	gatgaaacct	ttaagactcg	3600
tgaagctatt	cagcatgaat	caggaaatctt	gggaccttta	ctttatgggg	aagtgggaga	3660
cacactgttg	attatattta	agaatcaagc	aagcagacca	tataacatct	accctcacgg	3720
aatcactgat	gtccgtcctt	tgtattcaag	gagattacca	aaagggtgaa	aacatttgaa	3780
ggattttcca	attctgccag	gagaaatatt	caaataataa	tggacagtga	ctgtagaaga	3840
tgggccaact	aaatcagatc	ctcggtgctt	gacccgctat	tactctagtt	tcgttaatat	3900
ggagagagat	ctagcttcag	gactcattgg	ccctctcttc	atctgctaca	aagaatctgt	3960
agatcaaaga	ggaaaccaga	taatgtcaga	caagaggaa	gtcatcctgt	tttctgtatt	4020
tgatgagaac	cgaagctggt	acctcacaga	gaatatacaa	cgctttctcc	ccaatccagc	4080
tggagtgcag	cttgaggatc	cagagttcca	agcctccaac	atcatgcaca	gcatcaatgg	4140
ctatgttttt	gatagtttgc	agttgtcagt	ttgtttgcat	gaggtggcat	actggtacat	4200
tctaagcatt	ggagcacaga	ctgacttctt	ttctgtcttc	ttctctggat	ataccttcaa	4260
acacaaaatg	gtctatgaag	acacactcac	cctattccca	ttctcaggag	aaactgtctt	4320

-continued

catgtcga	gaaaaccag	gtctatggat	tctggggtgc	cacaactcag	actttcggaa	4380
cagaggcatg	accgccttac	tgaagggttc	tagttgtgac	aagaacactg	gtgattatta	4440
cgaggacagt	tatgaagata	tttcagcata	cttgctgagt	aaaaacaatg	ccattgaacc	4500
aagaagcttc	tcccagaatt	caagacaccc	tagcactagg	caaaagcaat	ttaatgccac	4560
cacaccacca	gtcttgaaac	gccatcaacg	cgaaataact	cgtactactc	ttcagtcaga	4620
tcaagaggaa	attgactatg	atgataccat	atcagttgaa	atgaagaagg	aagattttga	4680
catttatgat	gaggatgaaa	atcagagccc	cgcagcttt	caaaagaaaa	cacgacacta	4740
ttttattgct	gcagtgagga	ggctctggga	ttatgggatg	agtagctccc	cacatgttct	4800
aagaaacagg	gctcagagtg	gcagtgctcc	tcagttcaag	aaagttgttt	tccaggaatt	4860
tactgatggc	tcctttactc	agcccttata	cgtggagaa	ctaaatgaac	atttgggact	4920
cctggggcca	tatataagag	cagaagttga	agataaatatc	atggtaactt	tcagaaatca	4980
ggcctctcgt	ccctattcct	tctattctag	ccttatttct	tatgaggaag	atcagaggca	5040
aggagcagaa	cctagaaaaa	actttgtcaa	gcctaataag	accaaactt	acttttgtaa	5100
agtgaacat	catatggcac	ccactaaaga	tgagtttgac	tgcaaagcct	gggcttattt	5160
ctctgatgtt	gacctggaaa	aagatgtgca	ctcaggcctg	attggacccc	ttctggtctg	5220
ccacactaac	acactgaacc	ctgctcatgg	gagacaagtg	acagtacagg	aatttgctct	5280
gtttttcacc	atctttgatg	agacccaaag	ctgggtacttc	actgaaaata	tggaaagaaa	5340
ctgcagggct	ccctgcaata	tccagatgga	agatcccact	tttaaagaga	attatcgctt	5400
ccatgcaatc	aatggctaca	taatggatag	actacctggc	ttagtaatgg	ctcaggatca	5460
aaggattcga	tggatctcgc	tcagcatggg	cagcaatgaa	aacatccatt	ctattcattt	5520
cagtggaacat	gtgttccactg	tacgaaaaaa	agaggagtat	aaaatggcac	tgtacaatct	5580
ctatccaggt	gtttttgaga	cagtggaat	gttaccatcc	aaagctggaa	tttggcgggt	5640
ggaatgcctt	attggcgagc	atctacatgc	tgggatgagc	acactttttc	tgggtgtacag	5700
caataagtgt	cagactcccc	tgggaatggc	ttctggacac	attagagatt	ttcagattac	5760
agcttcagga	caatatggac	agtgggcccc	aaagctggcc	agacttcatt	attccggatc	5820
aatcaatgcc	tggagcacca	aggagccctt	ttcttgatc	aaggtggatc	tgttggcacc	5880
aatgattatt	cacggcatca	agaccagggg	tgcccgctcag	aagttctcca	gcctctacat	5940
ctctcagttt	atcatcatgt	atagttctga	tgggaagaag	tggcagactt	atcagggaaa	6000
ttccactgga	accttaaatg	tcttctttgg	caatgtggat	tcactctggga	taaaacacaa	6060
tatttttaac	cctccaatta	ttgctcgata	catccgtttg	cacccaactc	attatagcat	6120
tcgcagcact	cttcgcatgg	agttgatggg	ctgtgattta	aatagttgca	gcatgccatt	6180
gggaatggag	agtaaagcaa	tatcagatgc	acagattact	gcttcaccc	actttaccaa	6240
tatgtttgcc	acctggtctc	cttcaaaaag	tcgacttcac	ctccaaggga	ggagtaatgc	6300
ctggagacct	cagggtgaata	atccaaaaga	gtggctgcaa	gtggacttcc	agaagacaat	6360
gaaagtcaca	ggagtaacta	ctcagggagt	aaaatctctg	cttaccagca	tgtatgtgaa	6420
ggagttcctc	atctccagca	gtcaagatgg	ccatcagtg	actctctttt	ttcagaatgg	6480
caaagtaaag	gtttttcagg	gaaatcaaga	ctccttcaca	cctgtgggtga	actctctaga	6540
cccaccgtta	ctgactcgct	accttcgaat	tcacccccag	agttgggtgc	accagattgc	6600

-continued

cctgaggatg gaggttcttg gctgcgaggc acaggacctc tactgacaat tgacgctgat	6660
cagcctcgac tgtgccttct agttgccagc catctgttgt ttgcccctcc cccgtgcctt	6720
ccttgacctt ggaaggtgac actcccactg tcctttctta ataaaaatgag gaaattgcat	6780
cgcattgtct gagtagtgt cattctatct tgggggggtg ggtggggcag gacagcaagg	6840
gggaggattg ggaagacaat agcaggcatg ctggggagag atctaggaac ccctagtgat	6900
ggagttggcc actccctctc tgcgcgctcg ctgcgtcact gaggccgccc gggcaaagcc	6960
cggggtctcg gcgacctttg gtcgcccggc ctgagtgagc gagcgagcgc gcagagaggg	7020
agtggccaac cccccccccc cccccctgc aggcgattct cttgtttgct ccagactctc	7080
aggcaatgac ctgatagcct ttgtagagac ctctcaaaaa tagctacctt ctccggcatg	7140
aatttatcag ctagaacggt tgaatatcat attgatggtg atttgactgt ctccggcctt	7200
tctcaccctt ttgaatcttt acctacacat tactcaggca tgcatttaa aatatatgag	7260
ggttctaaaa atttttatcc ttgcgttgaa ataaaggctt ctcccgcaaa agtattacag	7320
ggtcataatg tttttggtac aaccgattta gctttatgct ctgaggcttt attgcttaat	7380
tttgctaatt ctttgctctg cctgtatgat ttattggatg ttggaattcc tgatgcggta	7440
ttttctcctt acgcctctgt gcggtatttc acaccgcata tgggtgacctc tcagtacaat	7500
ctgctctgat gccgcatagt taagccagcc ccgacaccgc ccaacaccgc ctgacgcgcc	7560
ctgacgggct tgtctgctcc cggcatccgc ttacagacaa gctgtgaccg tctccgggag	7620
ctgcatgtgt cagaggtttt caccgtcatc accgaaacgc gcgagacgaa agggcctcgt	7680
gatacgcta tttttatagg ttaatgtcat gataataatg gtttcttaga cgtcaggtgg	7740
cacttttcgg ggaatgtgc gcggaacccc tatttgttta tttttctaaa tacattcaaa	7800
tatgtatccg ctcatgagac aataaccctg ataaatgctt caataatatt gaaaaaggaa	7860
gagtatgagt attcaacatt tccgtgtcgc ccttattccc ttttttgcgg cattttgcct	7920
tctgttttt gctcaccag aaacgctggt gaaagtaaaa gatgctgaag atcagttggg	7980
tgcacgagtg gggtacatcg aactggatct caacagcggg aagatccttg agagttttcg	8040
ccccgaagaa cgttttccaa tgatgagcac ttttaaagtt ctgctatgtg gcgcgggtatt	8100
atcccgatatt gacgcggggc aagagcaact cggtcggcgc atacactatt ctccagaatga	8160
cttggttgag tactcaccag tcacagaaaa gcactcttac gatggcatga cagtaagaga	8220
attatgcagt gctgccataa ccatgagtga taacactgcg gccaaactac ttctgacaa	8280
gatcggagga ccgaaggagc taaccgcttt tttgcacaac atgggggatc atgtaactcg	8340
ccttgatcgt tgggaaccgg agctgaatga agccatacca aacgacgagc gtgacaccac	8400
gatgcctgta gcaatggcaa caacgttgcg caaactatta actggcgaac tacttactct	8460
agcttcccg caacaattaa tagactggat ggaggcggat aaagtgcag gaccacttct	8520
gcgctcgcc cttccggctg gctggtttat tgctgataaa tctggagccg gtgagcgtgg	8580
gtctcgcggt atcattgcag cactggggcc agatggtaag ccctcccgta tcgtagtatt	8640
ctacacgacg gggagtcagg caactatgga tgaacgaaat agacagatcg ctgagatagg	8700
tgctcactg attaagcatt ggtaactgtc agaccaagtt tactcatata tacttttagat	8760
tgatttaaaa cttcattttt aatttaaaag gatctagggtg aagatccttt ttgataatct	8820
catgacaaa atcccttaac gtgagttttc gttccactga gcgtcagacc ccgtagaaaa	8880

-continued

gatcaaagga tcttcttgag atcctttttt tctgcgcgta atctgctgct tgcaaacaaa	8940
aaaaccaccg ctaccagcgg tggtttggtt gccggatcaa gagctaccaa ctctttttcc	9000
gaaggtaact ggcttcagca gagcgcagat accaaatact gtccttctag tgtagccgta	9060
gttaggccac cacttcaaga actctgtagc accgcctaca tacctcgtc tgctaactct	9120
gttaccagtg gctgctgcca gtggcgataa gtcgtgtctt accgggttgg actcaagacg	9180
atagttaccg gataaggcgc agcggtcggg ctgaacgggg gggtcgtgca cacagcccag	9240
cttgagcgca acgacctaca ccgaactgag atacctacag cgtgagctat gagaaagcgc	9300
cacgcttccc gaaggggagaa agggcgacag gtatccggta agcggcaggg tcggaacagg	9360
agagcgacg agggagcttc cagggggaaa cgctggtat ctttatagtc ctgtcgggtt	9420
tcgccacctc tgacttgagc gtcgattttt gtgatgctcg tcaggggggc ggagcctatg	9480
gaaaaacgcc agcaacgcgg cctttttacg gttcctggcc ttttctggc cttttgctca	9540
catgttcttt cctgcgttat cccctgattc tgtggataac cgtattaccg cctttgagtg	9600
agctgatacc gctcgccgca gccgaacgac cgagcgcagc gagtcagtga gcgaggaagc	9660
ggaagagcgc ccaatacgca aaccgcctct cccgcgcgt tggccgattc attaatagcag	9720

<210> SEQ ID NO 5

<211> LENGTH: 7757

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: pAAV-CB-hHC1690-X10

<400> SEQUENCE: 5

aattcccatc atcaataata taccttattt tggattgaag ccaatatgat aatgaggggg	60
tggagtttgt gacgtggcgc ggggcgtggg aacggggcgg gtgacgtagt agtctctaga	120
ggccccagc gaccttgacg ggcatctgcc cggcatttct gacagctttg tgaactgggt	180
ggccgagaag gaatgggagt tgccgccaga ttctgacatg gatctgaatc tgattgagca	240
ggcaccctg accgtggcgg agaagctgca tcgctggcgt aatagcgaag aggcccgac	300
cgatcgccct tcccaacagt tgccgagcct gaatggcgaa tgggaattcca gacgattgag	360
cgtcaaaatg taggtatttc catgagcgtt tttcctgttg caatggctgg cggtaatat	420
gtttctggata ttaccagcaa ggccgatagt ttgagttctt ctactcaggc aagtgatgtt	480
attactaatc aaagaagtat tgcgacaacg gtttaatttc gtgatggaca gactctttta	540
ctcggtggcc tcaactgatta taaaaacact tctcaggatt ctggcgtacc gttcctgtct	600
aaaaatccct taatcggcct cctgttttag tcccgtcttg attctaacga ggaaagcacg	660
ttatacgtgc tcgtcaaagc aaccatagta cgcgccctgt agcggcgcgt taagcgcggc	720
gggtgtgggt gttacgcgca gcgtgaccgc tacacttgcc agcgccttag cgcgcgtcc	780
tttcgtttt ttccttctt tctcgcac gttcgcggc tttcccgtc aagctctaaa	840
tcgggggctc cctttagggt tccgatttag tgctttacgg cacctcgacc ccaaaaaact	900
tgattagggt gatggttcac gtatggggc atcgccctga tagacggttt tcgcccctt	960
gacgttgag tccacgttct ttaatagtgg actctgttc caaactggaa caacactcaa	1020
ccctatctcg gtctattctt ttgatttata agggattttg ccgatttcgg cctattgggt	1080
aaaaaatgag ctgatttaac aaaaatttaa cgcgaatttt aacaaaatat taacgtttac	1140

-continued

aatttaaata	tttgcttata	caatcttct	gtttttggg	ctttctgat	tatcaaccg	1200
ggtacatatg	attgacatgc	tagttttacg	attaccgttc	atcgccctgca	gggggggggg	1260
gggggggggt	ggccactccc	tctctgcgcg	ctcgcctcgt	cactgaggcc	gggcgaccaa	1320
aggtcgcccc	acgcccgggc	tttgcccggg	cggcctcagt	gagcgagcga	gcgcgcagag	1380
agggagtggc	caactccatc	actaggggtt	cctagatctg	aattcgggtac	gtacctctgg	1440
tcgttacata	acttacggta	aatggcccgc	ctggctgacc	gcccacgac	ccgcccatt	1500
gacgtcaata	atgacgtatg	ttcccatagt	aacgccaata	gggactttcc	attgacgtca	1560
atgggtggag	tatttacggt	aaactgccc	cttgccagta	catcaagtgt	atcatatgcc	1620
aagtacgccc	cctattgacg	tcaatgacgg	taaatggccc	gcctggcatt	atgccagta	1680
catgacctta	tggaactttc	ctacttggca	gtacatctac	tcgaggccac	gttctgcttc	1740
actctcccca	tctccccccc	ctccccacc	ccaattttgt	atttatttat	tttttaatta	1800
ttttgtgcag	cgatgggggc	gggggggggg	gggggggggg	cgcgcgccag	gcggggcggg	1860
gcggggcgag	gggcgggggc	gggcgaggcg	gagagggtcg	gcggcagcca	atcagagcgg	1920
cgcgctccga	aagtttcctt	ttatggcgag	gcggcgggcg	cggcggccct	ataaaaagcg	1980
aagcgcgcgg	cgggcggggg	cgggatcagc	caccgcgggtg	gcggcctaga	gtcgacgagg	2040
aactgaaaa	ccagaaagt	aactggtaag	tttagtcttt	ttgtctttta	tttcaggctc	2100
cggatccggt	ggtggtgcaa	atcaagaac	tgctcctcag	tggtatgtgc	ctttacttct	2160
aggcctgtac	ggaagtgtta	cttctgctct	aaaagctcgc	gaattgtacc	cgcggccgct	2220
tttcaaatg	caaatagagc	tctccacctg	cttctttctg	tgctttttgc	gattctgctt	2280
tagtgccacc	agaagatact	acctgggtgc	agtggaaactg	tcattgggact	atatgcaaag	2340
tgatctcggt	gagctgcctg	tggaacgcaag	atttctctct	agagtgcaca	aatcttttcc	2400
attcaacacc	tcagtcgtgt	acaaaaagac	tctgtttgta	gaattcacgg	atcacctttt	2460
caacatcgct	aagccaaggc	cacctgggat	gggtctgcta	ggctctacca	tccaggctga	2520
ggtttacgac	acggtggtcg	ttacctgaa	gaacatggct	tctcatcccg	ttagtcttca	2580
cgtgtcggc	gtctccttct	ggaaatcttc	cgaaggcgct	gaatatgagg	atcacaccag	2640
ccaaagggag	aaggaagacg	ataaagtctt	tcccggtaaa	agccaaacct	acgtctggca	2700
ggctctgaaa	gaaaatggtc	caacagctct	tgaccacca	tgtcttacct	actcatacct	2760
gtctcacgtg	gaactgggtga	aagacctgaa	ttcgggcctc	attggagccc	tactagtatg	2820
tagagaaggg	agtctggcca	aggaaaagac	acagaccttg	cacaaattta	tactactttt	2880
tgctgtattt	gatgaaggga	aaagtggca	ctcagaaaca	aagaactcct	tgatgcagga	2940
tagggatgct	gcattctgct	gggcctggcc	taaaatgcac	acagtcaatg	gttatgtaaa	3000
cagggtctctg	ccaggctctga	ttggatgcca	caggaaatca	gtctattggc	atgtgattgg	3060
aatgggcacc	actcctgaag	tgactcaat	attcctcgaa	ggtcacacat	ttcttctgag	3120
gaaccatcgc	caggcgctct	tggaatctc	gccataaact	ttccttactg	ctcaaacact	3180
cttgatggac	cttgacaggt	ttctactgtt	ttgtcatatc	tcttcccacc	aacatgatgg	3240
catggaagct	tatgtcaaa	tagacagctg	tccagaggaa	ccccaaactac	gaatgaaaaa	3300
taatgaagaa	gcggaagact	atgatgatga	tcttactgat	tctgaaatgg	atgtggtcag	3360
gtttgatgat	gacaactctc	cttcctttat	ccaaattcgc	tcagttgcca	agaagcatcc	3420

-continued

taaaacttgg gtacattaca ttgctgctga agaggaggac tgggactatg ctcccttagt	3480
cctcgccccc gatgacagaa gttataaaaag tcaatatttg aacaatggcc ctcagcggat	3540
tggtaggaag tacaaaaaag tccgatttat ggcatacaca gatgaaacct ttaagactcg	3600
tgaagctatt cagcatgaat caggaatctt gggaccttta ctttatgggg aagttggaga	3660
cacactgttg attatattta agaatcaagc aagcagacca tataacatct accctcacgg	3720
aatcactgat gtccgcctt tgtattcaag gagattacca aaaggtgtaa aacatttgaa	3780
ggattttcca attctgccag gagaaatatt caaatataaa tggacagtga ctgtagaaga	3840
tgggccaact aaatcagatc ctcggtgcct gaccgcgtat tactctagtt tcgttaatat	3900
ggagagagat ctagcttcag gactcattgg cctctctctc atctgctaca aagaatctgt	3960
agatcaaaga ggaaaccaga taatgtcaga caagaggaat gtcacctctgt tttctgtatt	4020
tgatgagaac cgaagctggt acctcacaga gaatatacaa cgctttctctc ccaatccagc	4080
tggagtgcag cttgaggatc cagagttcca agcctccaac atcatgcaca gcatcaatgg	4140
ctatgttttt gatagtttgc agttgtcagt ttgtttgcat gaggtggcat actggtacat	4200
tctaagcatt ggagcacaga ctgacttcct ttctgtcttc ttctctggat atacctcaa	4260
acacaaaatg gtctatgaag acacactcac cctattccca ttctcaggag aaactgtctt	4320
catgtcgatg gaaaaccag gtctatggat tctgggggtgc cacaactcag actttcggaa	4380
cagaggcatg accgccttac tgaaggttct tagttgtgac aagaacctg gtgattatta	4440
cgaggacagt tatgaagata tttcagcata cttgctgagt aaaaaaatg ccattgaacc	4500
aagaagcttc tcccagaatc caccagtctt gaaacgcat caacgcgaaa taactcgtac	4560
tactcttcag tcagatcaag aggaaattga ctatgatgat accatatcag ttgaaatgaa	4620
gaaggaagat tttgacattt atgatgagga tgaaaatcag agccccgca gctagcaatt	4680
gtagtctgaa cgctgatcag cctcgactgt gccttctagt tgccagccat ctgttgtttg	4740
ccccccccc gtgccttctc tgaccctgga aggtgccact cccactgtcc ttctctaata	4800
aaatgaggaa attgcatcgc attgtctgag taggtgtcat tctattcttg ggggtgggt	4860
ggggcaggac agcaaggggg aggattggga agacaatagc aggcattgctg gggagagatc	4920
taggaacccc tagtgatgga gttggccact cctctctctc gcgctcgctc gctcactgag	4980
gccgccccgg caaagcccg gcgtcgggcg acctttggtc gcccggcctc agtgagcgag	5040
cgagcgcga gagaggaggt ggccaacccc ccccccccc cccctgcagg cgattctctt	5100
gtttgtctca gactctcagg caatgacctg atagcctttg tagagacctc tcaaaaatag	5160
ctacctctc cggcattgaat ttatcagcta gaacggttga atatcatatt gatggtgatt	5220
tgactgtctc cggcctttct caccgcttg aatctttacc tacacattac tcaggcattg	5280
catttaaaat atatgaggtg tctaaaaatt tttatccttg cgttgaaata aaggcttctc	5340
ccgcaaaagt attacaggtg cataatgttt ttggtacaac cgatttagct ttatgctctg	5400
aggctttatt gcttaatttt gctaattctt tgccttgctt gtatgattta ttggatgttg	5460
gaattctcga tgcggtatct tctccttacg catctgtgcg gtatttcaca ccgcatatgg	5520
tgcactctca gtacaatctg ctctgatgcc gcatagttaa gccagccccg acaccgcca	5580
acaccgctg acgcgcctg acgggcttgt ctgctcccg catccgctta cagacaagct	5640
gtgacgtct cggggagctg catgtgtcag aggttttcac cgtcatcacc gaaacgcgcg	5700

-continued

agacgaaagg gcctcgtgat acgcctat	ttatagg	tta	atg	cat	gat	aataat	ggtt	5760
tcttagacgt caggtggcac ttttcgggga	aatgtgcgcg	gaacccctat	ttgtttat	5820				
ttctaataac attcaaatat gtatcgcctc	atgagacaat	aacctgata	aatgcttcaa	5880				
taatattgaa aaaggaagag tatgagtatt	caacatttcc	gtgtcgccct	tattcccttt	5940				
tttgcggcat tttgccttcc tgtttttgct	caccagaaa	cgctggtgaa	agtaaaagat	6000				
gctgaagatc agttgggtgc acgagtgggt	tacatcgaac	tggatctcaa	cagcggtaag	6060				
atccttgaga gttttcgccc cgaagaacgt	tttccaatga	tgagcacttt	taaagttctg	6120				
ctatgtggcg cggtattatc cgtattgac	gccgggcaag	agcaactcgg	tcgccgcata	6180				
cactattctc agaatgactt ggttgagtac	tcaccagtea	cagaaaagca	tcttacggat	6240				
ggcatgacag taagagaatt atgcagtgct	gccataacca	tgagtataaa	caactgcggcc	6300				
aacttacttc tgacaacgat cggaggaccg	aaggagctaa	ccgctttttt	gcacaacatg	6360				
ggggatcatg taactcgctt tgatcgttgg	gaaccggagc	tgaatgaagc	cataccaaac	6420				
gacgagcgtg acaccacgat gcctgtagca	atggcaacaa	cggtgcgcaa	actattaact	6480				
ggcgaaactac ttactctagc ttcccggcaa	caattaatag	actggatgga	ggcggataaa	6540				
ggtgcaggac cacttctgcg ctccggcctt	ccggttggtt	ggttttattgc	tgataaatct	6600				
ggagccgggtg agcgtgggtc tcgcggtatc	attgcagcac	tggggccaga	tggttaagccc	6660				
ttccgtatcg tagttatcta cacgacgggg	agtcaggcaa	ctatggatga	acgaaataga	6720				
cagatcgctg agataggtgc ctactgatt	aagcattggt	aactgtcaga	ccaagtttac	6780				
tcatatatac tttagattga tttaaaactt	catttttaat	ttaaaaggat	ctaggtgaag	6840				
atcctttttg ataatctcat gacccaaatc	ccttaacgtg	agttttcgtt	ccactgagcg	6900				
tcagaccccg tagaaaagat caaaggatct	tcttgagatc	ctttttttct	gcgcgtaatc	6960				
tgctgcttgc aaacaaaaaa accacgccta	ccagcgggtg	tttgtttgcc	ggatcaagag	7020				
ctaccaactc tttttccgaa ggtaactggc	ttcagcagag	cgcagatacc	aaatactgtc	7080				
cttctagtgt agcctgagtt aggccaccac	ttcaagaact	ctgtagcacc	gcctacatac	7140				
ctcgctctgc taatcctgtt accagtggct	gctgccagtg	gcgataagtc	gtgtcttacc	7200				
gggttggtgact caagacgata gttaccggat	aaggcgcagc	ggtcgggctg	aacgggggggt	7260				
tcgtgcacac agcccagctt ggagcgaacg	acctacaccg	aactgagata	cctacagcgt	7320				
gagctatgag aaagcgccac gcttcccga	gggagaaaag	cggacaggta	tccggtaagc	7380				
ggcagggctg gaacaggaga gcgcacgagg	gagcttcag	ggggaacgc	ctggatatct	7440				
tatagtcctg tcgggtttcg ccacctctga	cttgagcgtc	gattttttgtg	atgctcgtea	7500				
ggggggcgga gcctatggaa aaacgccagc	aacgcggcct	ttttacgggt	cctggccttt	7560				
tgctggcctt ttgctcacat gttctttcct	gcgttatccc	ctgattctgt	ggataaacgt	7620				
attaccgcct ttgagtgagc tgataccgct	cgccgcagcc	gaacgaccga	gcgcagcgag	7680				
tcagtgagcg aggaagcgga agagcgccca	atacgcaaac	cgcctctccc	cgcgcgttgg	7740				
ccgattcatt aatgcag				7757				

<210> SEQ ID NO 6

<211> LENGTH: 7757

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

-continued

<223> OTHER INFORMATION: pAAV-CB-hHC1690-X5

<400> SEQUENCE: 6

```

aattcccatc atcaataata taccttattt tggattgaag ccaatatgat aatgaggggg 60
tggagtttgt gacgtggcgc ggggcgtggg aacggggcgg gtgacgtagt agtctctaga 120
ggccccagc gaccttgacg ggcacatgcc cggcatttct gacagctttg tgaactgggt 180
ggcgagaag gaatgggagt tgccgccaga ttctgacatg gatctgaatc tgattgagca 240
ggcaccctg accgtggcgc agaagctgca tcgctggcgt aatagcgaag aggcccgac 300
cgatcgccct tcccaacagt tgcgcagcct gaatggcgaa tggaattcca gacgattgag 360
cgtaaaatg taggtatttc catgagcgtt ttctctgttg caatggctgg cggtaatatt 420
gttctggata ttaccagcaa ggccgatagt ttgagttctt ctactcaggc aagtgatgtt 480
attactaatc aaagaagtat tgcgacaacg gtttaattgc gtgatggaca gactctttta 540
ctcggtggcc tcaactgatta taaaaacact tctcaggatt ctggcgtaac gttctgtct 600
aaaaaccctt taatcgccct cctgttttag tcccgcctcg attctaacga ggaaagcacg 660
ttatacgtgc tcgtcaaagc aaccatagta cgcgccctgt agcggcgcat taagcgcggc 720
gggtgtgggt gttacgcgca gcgtgaccgc tacacttgcc agcgccctag cgcccgctcc 780
tttcgctttc ttcccttctt ttctcgccac gttcgccggc tttcccgctc aagctctaaa 840
tcgggggctc cctttagggt tccgatttag tgctttacgg cacctcgacc ccaaaaaact 900
tgattagggt gatggttcac gtatggggcc atcgccctga tagacggttt ttgcgccttt 960
gacgttgagg tccacgttct ttaatagtgg actcttgctt caaactggaa caaactcaa 1020
ccctatctcg gtctattctt ttgatttata agggattttg ccgatttcgg cctattgggt 1080
aaaaaatgag ctgatttaac aaaaatttaa cgcgaatttt aacaaaatat taacgtttac 1140
aatttaata tttgcttata caatcttctt gtttttgggg cttttctgat tatcaaccgg 1200
ggtaacatag attgacatgc tagttttacg attaccgttc atcgccctga gggggggggg 1260
gggggggggt ggccactccc tctctgcgcg ctgcctcgct cactgaggcc gggcgaccaa 1320
aggtcgcccg acgcccgggc tttgcccggg cggcctcagt gagcgagcga gcgcgcagag 1380
agggagtggc caactccatc actaggggtt cctagatctg aattcggtac gtacctctgg 1440
tcgttacata acttaaggta aatggccgcg ctggctgacc gcccaacgac ccgcccatt 1500
gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc attgacgtca 1560
atgggtggag tatttacggt aaactgccca cttggcagta catcaagtgt atcatatgcc 1620
aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt atgccagta 1680
catgacctta tgggactttc ctacttgcca gtacatctac tcgaggccac gttctgcttc 1740
actctcccca tctccccccc ctccccaccc ccaattttgt atttatttat tttttaatta 1800
ttttgtgcag cgatgggggc gggggggggg gggggggggg cgcgcgccag gcggggcggg 1860
gcggggcgag gggcgggggc gggcgaggcg gagaggtgcg gcggcagcca atcagagcgg 1920
cgcgctccga aagtttctt ttatggcgag gcggcgggcg cggcgccctc ataaaaagcg 1980
aagcgcgcg cgggcgggag cgggatcagc caccgcggtg gcggcctaga gtcgacgagg 2040
aactgaaaaa ccagaaagt aactggtaag tttagtcttt ttgtctttta ttccaggtcc 2100
cggatccggt ggtggtgcaa atcaaagaac tgctcctcag tggatgttgc ctttacttct 2160

```

-continued

aggcctgtac ggaagtgtta cttctgtctt aaaagctgcg gaattgtacc cgcggccgct	2220
tttcaaatg caaatagagc tctccacctg cttctttctg tgccttttgc gattctgctt	2280
tagtgccacc agaagatact acctgggtgc agtggaactg tcattgggact atatgcaaag	2340
tgatctcggg gagctgcctg tggacgcaag atttctctct agagtgccaa aatcttttcc	2400
attcaacacc tcagtcgtgt acaaaaagac tctgtttgta gaattcacgg atcacctttt	2460
caacatcgct aagccaaggc caccctggat gggctctgcta ggtctacca tccaggtga	2520
ggtttatgat acagtggctg ttacacttaa gaacatggct tcccatcctg tcagtcttca	2580
tgctgttggt gtatctact ggaaatcttc tgaggagct gaatatgatg atcagaccag	2640
tcaaggagg aaagaagatg ataaagtctt cctgggtaaa agccatacat atgtctggca	2700
ggtctgaaa gagaatggct caacagcttc tgaccacca tgccttacct actcatatct	2760
ttctcatgtg gacctggtaa aagacttgaa ttcaggcttc attggagccc tactagtatg	2820
tagagaagg agtctggcca aggaaaagac acagacctg cacaattta tactactttt	2880
tgctgtattt gatgaaggga aaagtggca ctcagaaaca aagaactcct tgatgcagga	2940
tagggatgct gcatctgctc gggcctggcc taaaatgcac acagtcaatg gttatgtaa	3000
caggctctg ccaggtctga ttggatgcca caggaaatca gtctattggc atgtgattgg	3060
aatgggcacc actcctgaag tgcactcaat attcctcgaa ggtcacacat ttcttgtag	3120
gaaccatgc caggcgtcct tggaaatctc gccataact ttccttactg ctcaaacact	3180
cttgatggac cttggacagt ttctactgtt ttgtcatac tcttcccacc aacatgatgg	3240
catggaagct tatgtcaaag tagacagctg tccagaggaa cccaactac gaatgaaaa	3300
taatgaagaa gcggaagact atgatgatga tcttactgat tctgaaatgg atgtggtcag	3360
gtttgatgat gacaactctc cttcctttat ccaaattcgc tcagttgcca agaagcatcc	3420
taaaacttgg gtacattaca ttgctgctga agaggaggac tgggactatg ctcccttagt	3480
cctcgcccc gatgacagaa gttataaaag tcaatatttg aacaatggcc ctacagcgat	3540
tggtaggaag tacaaaaaag tccgatttat ggcatacaca gatgaaacct ttaagactcg	3600
tgaagctatt cagcatgaat caggaaatctt gggaccttta ctttatgggg aagttggaga	3660
cacactgttg attatattta agaatcaagc aagcagacca tataacatct accctcacgg	3720
aatcactgat gtccgtcctt tgtattcaag gagattacca aaagggtgaa aacatttgaa	3780
ggattttcca attctgccag gagaaatatt caaatataaa tggacagtga ctgtagaaga	3840
tgggccaact aaatcagatc ctcggtgcct gaccgctat tactctagtt tcgttaatat	3900
ggagagagat ctagcttcag gactcattgg cctctctctc atctgctaca aagaatctgt	3960
agatcaaaga ggaaaccaga taatgtcaga caagaggaa gtcacacctgt tttctgtatt	4020
tgatgagaac cgaagctggt acctcacaga gaatatacaa cgctttctcc ccaatccagc	4080
tggagtgcag cttgaggatc cagagttcca agcctccaac atcatgcaca gcatcaatgg	4140
ctatgttttt gatagtttgc agttgtcagt ttgtttgcat gaggtggcat actggtacat	4200
tctaagcatt ggagcacaga ctgacttctt ttctgtcttc ttctctggat atacctcaa	4260
acacaaaatg gtctatgaag acacactcac cctattccca ttctcaggag aaactgtctt	4320
catgtcgatg gaaaaccag gtctatggat tctgggtgct cacaactcag actttcgga	4380
cagaggcatg accgccttac tgaagggttc tagttgtgac aagaactctg gtgattatta	4440

-continued

cgaggacagt tatgaagata tttcagcata cttgctgagt aaaaacaatg ccattgaacc	4500
aagaagcttc tcccagaatc caccagtctt gaaacgccat caacgcgaaa taactcgtac	4560
tactcttcag tcagatcaag aggaaattga ctatgatgat accatatcag ttgaaatgaa	4620
gaaggaagat ttgacattt atgatgagga tgaaaatcag agcccccgca gctagcaatt	4680
gtagtctgaa cgtgatcag cctcgactgt gccttctagt tgccagccat ctgttgtttg	4740
ccccccccc gtgccttctc tgaccctgga aggtgccact cccactgtcc ttctctaata	4800
aatgaggaa attgcatcgc attgtctgag taggtgtcat tctattctgg ggggtggggt	4860
ggggcaggac agcaaggggg aggattggga agacaatagc aggcattgctg gggagagatc	4920
taggaacccc tagtgatgga gttggccact cctctctgc gcgtcgtc gctcactgag	4980
gccgcccggg caaagcccgg gcgtcgggag acctttggtc gcccgccctc agtgagcgag	5040
cgagcgcga gagaggaggt ggccaacccc ccccccccc cccctgcagg cgattctctt	5100
gtttgtcca gactctcagg caatgacctg atagcctttg tagagacctc taaaaaatag	5160
ctaccctctc cggcatgaat ttatcagcta gaacgggtga atatcatatt gatggtgatt	5220
tgactgtctc cggcctttct caccgctttg aatctttacc tacacattac tcaggcattg	5280
catttaaaat atatgagggt tctaaaaatt tttatccttg cgttgaaata aaggcttctc	5340
ccgcaaaagt attacagggt cataatgttt ttggtacaac cgatttagct ttatgctctg	5400
aggctttatt gcttaatttt gctaattctt tgccttgct gtatgattta ttggatgttg	5460
gaattcctga tgcggtattt tctccttacg catctgtcgg gtatttcaca ccgcataatg	5520
tgcactctca gtacaatctg ctctgatgcc gcatagttaa gccagccccg acaccgccca	5580
acaccgctg acgcgccctg acgggcttgt ctgctcccg catccgctta cagacaagct	5640
gtgaccgtct cgggagctg catgtgtcag aggttttcac cgtcatcacc gaaacgcgcg	5700
agacgaaagg gcctcgtgat acgcctattt ttataggta atgtcatgat aataatggtt	5760
tcttagacgt cagggtggac ttttcgggga aatgtgcgcg gaaccctat ttgtttattt	5820
ttctaaatac attcaaatat gtatccgctc atgagacaat aacctgata atgcttcaa	5880
taatattgaa aaaggaagag tatgagtatt caacatttcc gtgtcgcctc tattcccttt	5940
tttgcgcat tttgccttcc tgtttttgct caccagaaa cgctggtgaa agtaaaagat	6000
gctgaagatc agttgggtgc acgagtgggt tacatcgaac tggatctcaa cagcggtaa	6060
atccttgaga gttttcgcgc cgaagaacgt tttccaatga tgagcacttt taaagtctg	6120
ctatgtggcg cggattatc cgtattgac gccgggcaag agcaactcgg tcgccgcata	6180
cactattctc agaatgactt ggttgagtac tcaccagtca cagaaaagca tcttacggat	6240
ggcatgacag taagagaatt atgcagtgtc gccataacca tgagtataa cactgcggcc	6300
aacttacttc tgacaacgat cggaggaccg aaggagctaa ccgctttttt gcacaacatg	6360
ggggatcatg taactcgcct tgatcgttgg gaaccggagc tgaatgaagc cataccaaac	6420
gacgagcgtg acaccacgat gcctgtagca atggcaacaa cgttgcgcaa actattaact	6480
ggcgaactac ttactctagc ttcccgcaa caattaatag actggatgga ggcggataaa	6540
gttgaggac cacttctgcg ctcgccctt ccggtggct ggtttattgc tgataaatct	6600
ggagccggtg agcgtgggtc tcgcggtatc attgcagcac tggggccaga tggtaagccc	6660
tcccgatcg tagttatcta cacgacgggg agtcaggcaa ctatggatga acgaaataga	6720

-continued

```

cagatcgctg agataggtgc ctcaactgatt aagcattggt aactgtcaga ccaagtttac 6780
tcatatatac tttagattga tttaaaactt catttttaat ttaaaaggat ctagggtgaag 6840
atcctttttg ataatctcat gaccaaaatc ccttaacgtg agttttcggt ccactgagcg 6900
tcagaccccg tagaaaagat caaaggatct tcttgagatc ctttttttct gcgcgtaatc 6960
tgctgcttgc aaacaaaaaa accacgcgta ccagcgggtg tttgtttgcc ggatcaagag 7020
ctaccaactc tttttccgaa ggtaactggc ttcagcagag cgcagatacc aaatactgtc 7080
cttctagtgt agccgtagtt aggccaccac ttcaagaact ctgtagcacc gcctacatac 7140
ctcgctctgc taatcctggt accagtggct gctgccagtg gcgataagtc gtgtcttacc 7200
gggttggaact caagacgata gttaccggat aaggcgcagc ggtcgggctg aacggggggt 7260
tcgtgcacac agcccagctt ggagcgaacg acctacaccg aactgagata cctacagcgt 7320
gagctatgag aaagcggcac gcttcccga gggagaaaagg cggacaggta tccggtaagc 7380
ggcagggctg gaacaggaga gcgcacgagg gagcttcag ggggaaacgc ctggtatctt 7440
tatagtctg tcgggtttcg ccacctctga cttgagcgtc gatttttgtg atgctcgtca 7500
ggggggcgga gcctatggaa aaacgccagc aacgcggcct ttttacggtt cctggccttt 7560
tgctggcctt ttgtcaccat gttctttcct gcgttatccc ctgattctgt ggataaccgt 7620
attaccgcct ttgagtgage tgataccgct cgccgcagcc gaacgaccga gcgcagcgag 7680
tcagtgagcg aggaagcgga agagcgccca atacgcaaac cgctctctcc cgcgcggttg 7740
ccgattcatt aatgcag 7757

```

```

<210> SEQ ID NO 7
<211> LENGTH: 200
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

```

```

<400> SEQUENCE: 7

```

```

Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser Trp Asp Tyr
1      5      10      15
Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg Phe Pro Pro
20     25     30
Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val Tyr Lys Lys
35     40     45
Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile Ala Lys Pro
50     55     60
Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val
65     70     75     80
Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser His Pro Val
85     90     95
Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala
100    105    110
Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys Val
115    120    125
Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu Lys Glu Asn
130    135    140
Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu Ser
145    150    155    160
His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala Leu
165    170    175

```

-continued

Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
180 185 190

His Lys Phe Ile Leu Leu Phe Ala
195 200

<210> SEQ ID NO 8
<211> LENGTH: 200
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 8

Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser Trp Asp Tyr
1 5 10 15

Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg Phe Pro Pro
20 25 30

Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val Tyr Lys Lys
35 40 45

Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile Ala Lys Pro
50 55 60

Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val
65 70 75 80

Tyr Asp Thr Val Val Val Thr Leu Lys Asn Met Ala Ser His Pro Val
85 90 95

Ser Leu His Ala Val Gly Val Ser Phe Trp Lys Ser Ser Glu Gly Ala
100 105 110

Glu Tyr Glu Asp His Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys Val
115 120 125

Leu Pro Gly Lys Ser Gln Thr Tyr Val Trp Gln Val Leu Lys Glu Asn
130 135 140

Gly Pro Thr Ala Ser Asp Pro Pro Cys Leu Thr Tyr Ser Tyr Leu Ser
145 150 155 160

His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala Leu
165 170 175

Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
180 185 190

His Lys Phe Ile Leu Leu Phe Ala
195 200

<210> SEQ ID NO 9
<211> LENGTH: 200
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 9

Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser Trp Asp Tyr
1 5 10 15

Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg Phe Pro Pro
20 25 30

Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val Tyr Lys Lys
35 40 45

Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile Ala Lys Pro
50 55 60

Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val
65 70 75 80

-continued

Tyr	Asp	Thr	Val	Val	Val	Thr	Leu	Lys	Asn	Met	Ala	Ser	His	Pro	Val
				85					90					95	
Ser	Leu	His	Ala	Val	Gly	Val	Ser	Tyr	Trp	Lys	Ser	Ser	Glu	Gly	Ala
			100					105					110		
Glu	Tyr	Asp	Asp	Gln	Thr	Ser	Gln	Arg	Glu	Lys	Glu	Asp	Asp	Lys	Val
		115					120					125			
Phe	Pro	Gly	Lys	Ser	His	Thr	Tyr	Val	Trp	Gln	Val	Leu	Lys	Glu	Asn
		130				135					140				
Gly	Pro	Thr	Ala	Ser	Asp	Pro	Pro	Cys	Leu	Thr	Tyr	Ser	Tyr	Leu	Ser
	145				150					155				160	
His	Val	Asp	Leu	Val	Lys	Asp	Leu	Asn	Ser	Gly	Leu	Ile	Gly	Ala	Leu
			165					170						175	
Leu	Val	Cys	Arg	Glu	Gly	Ser	Leu	Ala	Lys	Glu	Lys	Thr	Gln	Thr	Leu
		180						185					190		
His	Lys	Phe	Ile	Leu	Leu	Phe	Ala								
		195				200									

What is claimed is:

1. An isolated human factor VIII polypeptide mutant comprising one or more amino acid substitution(s) selected from the group consisting of I86, Y105, A108, D115, Q117, F129, G132, H134, M147 and L152.

2. The mutant of claim 1, wherein the one or more amino acid substitution(s) are selected from the group consisting of I86V, Y105F, A108S, D115E, Q117H, F129L, G132K, H134Q, M147T and L152P.

3. The mutant of claim 1, comprising amino acid substitutions in each of amino acids I86, Y105, A108, D115, Q117, F129, G132, H134, M147 and L152.

4. The mutant of claim 3, comprising each of the amino acid substitutions I86V, Y105F, A108S, D115E, Q117H, F129L, G132K, H134Q, M147T and L152P.

5. The mutant of claim 1, wherein the one or more amino acid substitution(s) are selected from the group consisting of I86, A108, G132, M147 and L152P.

6. The mutant of claim 5, wherein the one or more amino acid substitution(s) are selected from the group consisting of I86V, A108S, G132K, M147T and L152P.

7. The mutant of claim 5, comprising amino acid substitutions in each of amino acids I86, A108, G132, M147 and L152.

8. The mutant of claim 7, comprising amino acid substitutions in each of amino acids I86V, A108S, G132K, M147T and L152P.

9. The mutant of any one of claims 1 to 8, further comprising a deletion in the B domain of human factor VIII.

10. The mutant of claim 9, further comprising the a2 and/or a3 domain(s) of human factor VIII.

11. An isolated polynucleotide encoding the mutant of any one of claims 1 to 10.

12. An expression vector comprising the polynucleotide of claim 11.

13. A host cell comprising the polynucleotide of claim 11.

14. A host cell comprising the expression vector of claim 12.

15. A pharmaceutical composition comprising the expression vector of claim 14.

16. A method for treating a patient with a factor VIII deficiency comprising:

administering to the patient in need thereof the pharmaceutical composition of claim 15 in an amount effective for treating the factor VIII deficiency.

17. A method for expressing a human factor VIII polypeptide mutant comprising:

(a) transforming a host cell with an expression vector comprising the nucleic acid of claim 12;

(b) growing the host cell under conditions suitable for expressing the human factor VIII polypeptide mutant; and

(c) purifying the human factor VIII polypeptide mutant from host cells expressing said mutant.

* * * * *